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ERWIN F. SMITH
1854–1927

Erwin Frink Smith

1854-1927

It is a privilege to contribute to this JOURNAL something that may place on record an appreciation of Erwin F. Smith's contributions to bacteriological science in general and especially to American developments in this field. My personal acquaintance with him dates from the spring of 1889 when, although only a senior at the University of Michigan, I was privileged to attend Smith's doctor's examination, his dissertation dealing with peach yellows. He had proved it to be a transmissible disease but it clearly was not of fungous nature. What was the infectious principle? Was it possibly a bacterial pathogen? He soon learned that Adolph Mayer who studied the somewhat similar tobacco mosaic disease was likewise unable to find a causal organism but considered it to be of bacterial nature. Burrill had a decade earlier shown that the fire blight of pear was due to a bacterial pathogen. It is not a matter of record, so far as I know, but one who knew Smith's marvelously alert mind, keen intuitions, religiously patient thoroughness, can easily understand why he soon decided that he must perfect his bacteriological technique and learn all that was possible of the nature and relations of bacteria. Doubtless he was stimulated the more in this because following Burrill's earlier work (1878) and Arthur's more detailed studies (1885), showing how virulent and specific a parasite was *Bacillus amylovorus*, M. B. Waite, working as a close associate in the Department of Agriculture was proving the remarkable adaptations of this organism for such parasitic existence both because of its ability to spread in the presence of the natural plant acids and of the relation of insects to its dissemination and invasion through the nectaries of the pear flowers. He early established relations of close personal and professional nature with Theobald Smith, then director of the pathological

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laboratory of the national Bureau of Plant Industry and with Welch of Johns Hopkins University, two of the ablest American pioneers in medical bacteriology, and both men whose scholarship and scientific exactitude accorded so well with Smith's own exacting standards. The best evidence of a man's worth may often be found in his early and intimate friendships. These and others of like character, relating him to many scientific centers of the world, endured to the end of his life.

When Smith's critical mind in the early nineties reviewed the evidence concerning the possibility of bacteria being important causal factors in plant pathology he found a considerable suggestive comment, but, except for pear blight, a lack of reliable data in any way comparable to that from the related fields of animal pathology and of fermentation. The recently discovered facts as to the legume tubercle bacteria were, of course, also enough to stimulate such a mind as Smith's.

We all know the outcome, at least all the elder American bacteriologists do, and the younger ones should not be permitted to forget it. He saw the challenge and accepted it, with clear vision as to its magnitude. What is the rôle of bacteria in relation to plant diseases? In quick succession came his reports of progress toward the adequate answer.

His first paper (August, 1893) dealt with the bacterial wilt of cucurbits (cucumber, squash, cantaloupe), although he did not publish the technical description of the pathogen (*Bacillus tracheiphilus*) until 1895. There followed in close succession several publications on specific bacterial diseases with critical and detailed technical descriptions of the organisms respectively concerned which have been exacting models for all our younger American phytopathologists; that on the brown rot of tomato, potato and other Solanaceae (*Bacillus solanacearum*, 1896) that on the black rot of cabbage and other crucifers (*Pseudomonas campestre*,[1] 1897); on bean blight (*Bacillus phaseoli*, 1897); and on the bacterial wilt of maize (*Pseudomonas stewarti*, 1898).

[1] To accord with the historical purposes of this article it seems proper to use for each of the several organisms mentioned the binomial as first published by Smith, and this regardless of whether he may have used another name in his later publications.

Smith's observations and investigations thus early convinced him not only of the scientific and economic importance of bacterial diseases of plants, but also led him to foresee that persistent search would reveal an indefinitely great number of these[2]—a prediction which he lived to see amply verified. While he had already won the full confidence of his Amercian colleagues, no investigations of corresponding thoroughness had been made in this field in Europe.

Alfred Fischer of Berlin University in 1897, evidently after quite superficial examination of the literature, scouted the evidence of the occurrence of bacterial diseases of plants and instead concluded on *a priori* grounds that bacteria could not penetrate or persist in plant tissues as parasites. Smith, with characteristic promptness and fierce thoroughness, replied in the *Centralblatt f. Bakteriologie*, detailing evidence that not only won his case, but established his world leadership in this field of bacteriology. Although Smith thereafter continued in conjunction with his younger laboratory associates to search out and describe further new species of bacterial plant pathogens, his most important contributions were along two more comprehensive lines.

The first of these involved the comparative consideration of known bacterial plant pathogens. This actually began with his review of literature in the *American Naturalist* (1896–7) and was further advanced with his studies of certain yellow vascular parasites in 1901. The culmination came in his exhaustive treatises on Bacteria in Relation to Plant Disease, of which three quarto volumes had been published and the manuscript of others advanced before his death. The second of his major contributions concerned his studies of bacterial crown galls or plant tumors involving comparisons with malignant tumors of animals. Since my personal contacts were less intimate with Smith in these later undertakings I have asked his trusted laboratory associate, Frederick V. Rand, to supplement what I have said, especially upon these two matters.

L. R. JONES.

[2] Fortunately Smith gave us, beginning in 1896, an adequate picture of the status of knowledge of bacterial diseases of plants together with his vision of the real significance of this group of pathogens in a series of literature reviews in the *American Naturalist*.

At the time when Erwin F. Smith began his actual studies of the bacteria of plant disease (1893) he encountered a paucity of reliable information on the subject. Ten years later a considerable literature had grown up, much of it uncritical and nowhere gathered into one place. It seemed to him fitting, therefore, to attempt a well-digested summary of preceding work, including his own, in order to clear the air, to set the new science on a firm foundation, and to assist the newer workers in the use of sound methods of procedure. The Carnegie monograph series (Bacteria in Relation to Plant Disease) was thus written primarily for plant pathologists but also in the hope that it would prove useful to human and animal pathologists for purposes of comparison at a time when but little concerning the new plant pathology was known by outside interests.

Smith's expressed course had been to supplement his own general botanical studies with all that could be obtained by further training and experience in the knowledge of bacteriological methods; then to gather together and properly digest the world's literature of the subject; and finally "to carefully work over, in the laboratory, field, and greenhouse, as opportunity offered, all the so-called bacterial diseases of plants, submitting each supposed parasite to all the tests of modern pathology." In thus carrying out his purpose every disease discussed was carefully studied in his own laboratory in so far as material could be obtained.

In presenting to the public the first volume of the monograph series his characteristic modesty and caution were expressed in his quotation from Lavoisier: Man would never give anything to the public if he waited till he had reached the goal of his undertaking, which is ever appearing close at hand and yet slipping farther and farther as he draws nearer. "Those," he concludes, "who dwell in the clearer light of the next generation will build better than we have done and will scarcely realize how slowly and painfully many of us have groped about for what seems to them so plain."

In this monumental work Smith states that he has tried to point out at frequent intervals gaps in our knowledge, to express things

clearly and honestly, to distinguish between verified fact and speculation, and, finally, to leave each subject in somewhat better shape than he found it. The series begins with a full discussion of the plant disease and the causal organism as specific entities, the economic aspects of the subject and a full consideration of laboratory practice and procedure, the first volume concluding with a classified bibliography covering some sixty-four pages. The subject matter is continued in volume two by a history of plant bacteriology and its makers, followed here and in the succeeding volume by an exhaustive and detailed treatment of the vascular diseases of plants due to bacteria. The original plan called for two further subdivisions of the subject to include the parenchyma diseases without hyperplasia, and the cankers, tubercles and tumors. A considerable portion of this material was in manuscript form at the time of Smith's death. Fortunately some of the more essential points have been covered in his text-book, "Bacterial Diseases of Plants" (1920), but it is hoped that the full manuscript may be eventually worked over, brought up to date and published as one or more succeeding volumes to the series. It is noteworthy as indicating Smith's painstaking exactness that I find rubber-stamped on the title pages of volumes two and three, respectively, the following: "Copies of this book first issued Oct. 30, 1911," and "Aug. 4, 1914."

Following his early studies on the bacterial tubercle of olives published in 1904, he was struck by certain resemblances to a tumor of Paris daisy and forthwith initiated a study of the latter that was destined to lead him into many by-paths and to bring him more lasting recognition in the dual field of plant and animal pathology than all his other scientific work combined. The first two papers (Smith and Townsend, 1907), published both in English and German, concerned the etiology of crowngall. Then, during the subsequent years and up through 1926, came in rapid succession the long series of papers dealing with all phases of plant and animal cancers, bringing the grand total to some forty titles.

At first the etiology and control of the plant disease assumed first place in his mind. Then came his painstaking and exhaustive morphological studies followed soon by expression of his

ever-increasing conviction as to the striking resemblances between this disease and animal cancer. This stage culminated perhaps around 1916 in which year four out of his six papers dealt with comparisons between plant and animal cancers. There now followed his epoch-making studies of the mechanism of tumor formation, leading him finally into problems of histogenesis, and of formative stimuli and growth inhibitions in general, which are of such far reaching importance to the whole field of experimental biology. It was to these more fundamental phases that he gave most time and thought during the final years and months of his life. Indeed his last word here has not yet been said, as a number of posthumus papers will soon attest.

Perhaps one of the greatest and most lasting impressions from his scientific career is that given by his dogged perseverance in the life-long task of following out a problem in all its manifold ramifications and against all obstacles. His work and influence have left an indelible impression upon his age—and not alone upon plant science or upon animal science, but upon the whole field of experimental biology as well. As voiced at the funeral service by one of his life-long associates, "He has contributed his full share of effort to provide bread for the hungry ones of the world. He has done his part to heal their diseases. He has given to them words of beauty and inspiration!"

FREDERICK V. RAND.

TWENTY-NINTH ANNUAL MEETING OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

SCHOOL OF MEDICINE AND DENTISTRY, UNIVERSITY OF ROCHESTER, ROCHESTER, NEW YORK

December 28, 29, 30, 1927

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GENERAL BACTERIOLOGY

1. Physiology as a Basis for the Differentiation of Bacterial Genera. R. E. BUCHANAN, Department of Bacteriology, Iowa State University, Ames, Iowa.

A discussion of the contention of Dr. Ivan Hall and others that morphology (in the broad sense) should constitute the sole basis of differentiation of genera. It is concluded that a restriction of freedom in the choice of criteria for differentiation of genera is unwise, and would interfere seriously with a satisfactory natural grouping of microörganisms.

2. *Suggestions Concerning the Nomenclature of the Legume Root Nodule Bacteria.* I. L. Baldwin and E. B. Fred, University of Wisconsin, Madison, Wis.

In Bergey's Manual of Determinative Bacteriology (second edition, 1925) the root nodule bacteria of the Leguminosae are classified in the genus *Rhizobium* and two species are established. A thorough review of the literature in relation to the nomenclature of the microorganisms was presented at the twenty-eighth Annual Meeting of the Society of American Bacteriologists by Dr. Buchanan. Differentiation into the 2 species is established upon the basis of the morphology, flagellation, cultural characters, and habitat of the organisms.

Perhaps the most constant of the physiological properties of the root nodule bacteria is their ability to cause the formation of nodules upon certain species of the Leguminosae and not upon others. Certainly from the practical standpoint of the scientific worker, this is one of the most important characters of the organism. For the past several years, a utilitarian classification into "cross-inoculation" groups has been in use by students in this field. Recently with careful and detailed study of these organisms it has been shown that the members of each "cross-inoculation" group differ from those of each of the others, in some or all of the following points: morphology, cultural characters, physiological properties, and serological reactions.

In view of the fact that the root nodule organisms naturally fall into several groups, each of which presents definite and constant characters differentiating it from all other organisms, and because of the confusion arising from the present system of classification, it is proposed that several separate species be established. The genus, Rhizobium, is retained and the rules of the Bacteriological Code on Nomenclature, as adopted by the Society of American Bacteriologists, are followed in choosing species names. Species identification is based upon the morphology, cultural characters, physiological properties, and serological reactions of the bacteria. In each case where sufficient study has been made to render it possible, a species is established for each "cross-inoculation group."

3. *Nitrogen Fixation by Pure Cultures of Anaerobic Butyric Acid-forming Bacteria.* Elizabeth McCoy and W. M. Higby, University of Wisconsin, Madison, Wis.

A group of 46 anaerobic butyric acid bacteria have been used in studies of nitrogen fixation. The cultures include several species of

historical interest and also new strains isolated from cultivated soils. There are representatives of both butyl and butyric types; of the latter, there are three sub-types on the basis of general physiology and morphology.

The ability of these organisms to fix atmospheric nitrogen varies. From 0 to 3.0 mgm. of nitrogen per 100 cc. of culture under the conditions used were fixed. Thirty-three of these pure cultures have given definitely positive fixation. Ability to fix nitrogen does not belong to any one physiological group. Individual variations of the cultures and the following factors have been studied—temperature of fermentation, duration of fermentation, ratio of initial nitrogen to carbohydrate of the medium, rate of utilization of sugar and its relation to rate of nitrogen fixation.

4. *The Fixation of Nitrogen by Bacterium aerogenes and Related Species.* (To be read by title.) CHARLES E. SKINNER, Department of Bacteriology, University of Minnesota, Minneapolis, Minn.

Of 23 strains of *B. aerogenes* isolated from soil, milk, flour, and water, 3 strains were found which fixed atmospheric nitrogen, when grown in routine nitrogen free media. Not all strains of *B. radiobacter* were found to fix nitrogen. *B. aerogenes* or *B. radiobacter*, often both, were always encountered in the isolation of *Azotobacter* and the latter nearly always in the isolation of the nodule bacteria. *B. cloacae* was more often found in soil than *B. aerogenes*. No strains were found to fix nitrogen. *B. radicicola* is very closely related phylogenetically to *B. radiobacter* and *B. ærogenes*. The placing of the genus *Rhizobium* by Bergey among the autotrophic bacteria (family Nitrobacteriaceae) has no scientific justification. If maintained as a genus it belongs in or near the colon typhoid group. Need for further work on *B. radiobacter* is indicated.

5. *Study of Lactose Peptone Bile Media, with the Addition of Various Triphenylmethane Dyes, for the Detection of Members of the Colon Group. I. In Water Analysis.* CHARLES F. POE, J. T. FIELD, SIDNEY TOBEY AND NORMAN F. WITT. *II. In Milk Analysis.* CHARLES F. POE, WALTER H. HALE AND FRANCES E. POE. Department of Chemistry, University of Colorado, Boulder, Colo.

Different lactose peptone media, with the addition of bile varying from 0 per cent to 13 per cent and with the addition of various triphenylmethane dyes in amounts varying from 1 part in 100,000 to 1 part in 3500, were used. Each separate batch of the above media was

divided into 5 parts and each adjusted to a different pH, i.e., 6.0, 6.5, 7.0, 7.5, and 8.0

Beginning with the media containing the dyes in amount of 1 to 100,000 various lactose fermenting organisms, which do not belong to the colon-aerogenes groups but which might occur in water and milk, were tried. The percentage of bile and dye necessary to prevent the growth of these organisms was determined. The growth of the majority of these organisms was completely retarded by a dilution of 1 to 35,000. Starting with this dilution the various members of the colon-aerogenes groups were studied in the different media with the idea of determining the best percentages of bile and the various dyes for use in media for the presumptive test in the examination of milk and water.

6. The Fermentation of Glucose by Bacteria of the Genus Serratia. C. S. PEDERSON AND R. S. BREED, Agricultural Experiment Station, Geneva, N. Y.

A group of 6 cultures, found by an intensive bacteriological study to be representative of a large collection of cultures of the genus *Serratia*, was studied quantitatively with respect to their action on glucose. The purpose of this work was to secure information that would assist in differentiating the species in the genus and aid in working out the relationship between these organisms and those included in the colon-aerogenes-proteus group.

A 1 per cent Difco peptone, 3 per cent glucose medium was used for this study, the reaction being kept at neutrality by use of calcium carbonate. The flasks of media were incubated at 25°C. for one month. The first 2 cultures which are representative of the species *Serratia marcescens* Bizio (*Bacillus prodigiosus*) formed the following products: acetic, formic, succinic and levo-rotatory lactic acids, ethyl alcohol, acetyl methyl carbinol, butylene glycol, carbon dioxide and a trace of hydrogen. The 2 cultures proved to be almost identical in their fermentative powers.

The second pair, representative of the species *Serratia indica* Bergey were found to produce the same products with the exception of hydrogen, but in slightly different proportions.

The last pair, which according to numerous cultural tests varied but slightly from each other, were found to be entirely different. One of these, culture 261, which was received from the Hygienic Laboratory at Washington under the label *B. prodigiosus* and probably came originally from the Pasteur Institute, Paris, produced inactive lactic

acid, hydrogen in a greater proportion, as well as the other products named above. The second culture, K_6, came from the Kral collection under the label *B. rubrum* Kiel (*B. balticum*), and was isolated by Lubinsky, Pasteur Institute, Kiew. It varied in that it produced inactive lactic acid in a greater proportion, gave less carbon dioxide and more hydrogen than *S. marcescens*, and in this medium produced not more than a trace of acetyl methyl carbinol or butylene glycol.

7. *Studies in the Carbohydrate Metabolism of Ps. aeruginosa* (*B. pyocyaneus*). H. J. Sears, Department of Bacteriology, University of Oregon, Medical School, Portland, Oregon.

Determinations of pH, buffer and glucose content in cultures of *Ps. aeruginosa* in peptone solutions and in glucose peptone solutions containing concentrations of peptone from 0.1 to 5 per cent show that the extent of nitrogen metabolism by this organism has a profound effect on glucose metabolism. In cultures in which the peptone content is 2 per cent or more, glucose is utilized in large amounts, but the pH does not fall below neutrality, but rather soars to the limiting alkalinity. In concentrations below 2 per cent an acid reaction is generally produced and less glucose is used. In concentrations of peptone below 1 per cent the limiting acidity for the organism is reached in a few days and only small amounts of glucose are metabolised. Similar results are obtained where 0.3 per cent meat extract is added to the medium.

In mediums containing other sugars an acid reaction is not given even in solutions of peptone as low as 0.5 per cent. The pH curve does lie however consistently below that of a sugar free bouillon of the same composition when maltose, mannite and glycerine are used. By a different method it is shown that many substances other than glucose are utilizable by *Ps. aeruginosa* as a carbon source. In a medium consisting of 1 per cent ammonium tartrate and minute quantities of mineral salts this organism will not grow. When certain sugars and alcohols are added to this basic medium a good growth is obtained. Among such fermentable substances are, glucose, levulose, galactose, glycerine, mannitol, maltose, xylose, sorbitol and inositol. Lactose, saccharose and arabinose fail to stimulate growth.

Not only in its own cultures does the nitrogen metabolism of *Ps. aeruginosa* exert a profound effect on carbohydrate metabolism but when grown in symbiosis with such rapidly fermenting organisms as *B. coli* and *B. gasoformans*, a similar effect is seen in the increase of pH and sugar decomposition.

It is believed that the action of *Ps. aeruginosa* in all these cases is to keep the acidity down by means of alkaline substances formed in its very active metabolism of the nitrogenous constituents of the medium, thus permitting a longer carbohydrate splitting activity of the organism.

8. The Cell Wall a Factor in the Gram Reaction. VICTOR BURKE AND MILDRED BARNES, State College of Washington, Pullman, Washington.

The recent work of Stearns and Stearns has thrown some doubt on the cell wall as a factor in the Gram reaction. Their conclusions are at variance with those of Benians, who concluded that the cell wall is the dominant factor in determining the Gram reaction.

Experiments on the staining of Gram positive yeast cells indicated that with this organism the cell wall determines whether the protoplasm is to stain Gram-positive or Gram-negative. The use of indicators demonstrated that the cell wall acts directly in preventing decolorization rather than indirectly by controlling the hydrogen-ion concentration of the protoplasm. Extreme changes in hydrogen-ion concentration will affect the staining of yeast cells but within the range prevailing when the best Gram staining technique is employed the cell wall is the determining factor. Factors affecting the Gram reaction of bacterial cells have the same effect on the staining of yeast cells. Furthermore, experiments with the larger bacterial cells suggest that their staining is determined by the same factors that determine the staining of yeast cells. It is concluded that the cell wall is a factor in determining the Gram reaction.

9. Further Studies on a Modification of the Gram Stain. NICHOLAS KOPELOFF AND PETER COHEN, Psychiatric Institute, Ward's Island, N. Y.

Since the Kopeloff and Beerman modification of the Gram stain has found some favor at the hands of teachers and investigators, further attempts to improve this technic have been made. Testing a number of organisms with acetone and alcohol (95 per cent) separately and in various combinations, equal parts of these 2 decolorizing agents yields most satisfactory results. The length of time of application of the decolorizer is of importance and should be reduced to a minimum. With 0.1 per cent basic fuchsin as a counterstain, the time of application should be twenty to thirty seconds.

10. The Additive and Antagonistic Effect of Cations upon Bacterial Viability. C.-E. A. WINSLOW AND A. F. DOLLOFF, Department of Public Health, Yale School of Medicine, New Haven, Conn.

Investigations conducted during the last five years in the Yale laboratories have indicated that, so far as their fundamental effect upon bacterial viability is concerned, the various cations function only quantitatively and not qualitatively. All of them appear in a certain concentration to favor bacterial growth (probably by increasing cell permeability) and in a higher concentration to inhibit bacterial growth (probably by decreasing permeability and ultimate rupture of the cell membrane). The concentration at which a given effect is produced, however, differs widely.

The present study concerning the influence of sodium, potassium and magnesium upon bacterial growth in a simple synthetic medium indicates that many of the phenomena which have been described as antagonism may really be explained by the purely additive effects of the individual cations concerned. If we assume that magnesium is 8 times as effective as sodium or calcium in a given concentration, curves plotted to show the effect upon viability of different concentrations are essentially the same whether we use either one of the three salts alone or a combination of the salts. Even the hydrogen-ion appears to follow the same rule although its potency at a given concentration is of course very high.

The authors of course recognize that certain biochemical properties of bacteria as well as many properties of the cells of higher plants and animals may be affected by cations in a specific fashion which involves true qualitative antagonism. So far as the influence of cations upon bacterial viability is concerned, such true qualitative antagonism does not seem to be important.

11. Phase Reversal Within the Aqueous-Lipoidal System of the Bacterial Cell-Wall and its Relation to Spontaneous Agglutinability. RALPH R. MELLON, Western Pennsylvania Hospital, Pittsburgh, Pa.

The large number of studies on the electric charges of bacteria especially in its relation to spontaneous agglutination have never been able to evaluate the rôle of the surface tension factor. This, because of no satisfactory method for measuring bacterial interfacial tension, the method advocated by Northrup and DeKruif being quite inaccurate. The result has been an undue appreciation of the charge factor at the expense of the surface tension factor.

Several years ago one of our papers called attention to this aspect of

the situation. The ground for our position rested on experiments with sodium oleate whereby certain strains of high interfacial tension could be readily emulsified with this reagent. In this connection we also showed that the principle of ion antagonism was applicable, which indicated the presence of an aqueous-lipoid system in the cell-wall that was reversible by the cations Na and Ca where the anion Cl remained the same.

This contention is now susceptible of experimental demonstration with certain strains, *in which instances* the electric charge appears to play a minor or even an insignificant rôle in suspension stability. The experiments are as follows: The Ca ion brings the lipoid phase to the external surface of the bacterial cell-wall, as can be shown by the fact that the organism will pass from an aqueous "solvent" to lipoidal solvents. The effect of the Ca appears to be a maximal one, and the effect of the solvent a quantitative one, depending on the relative solubility of the lipoidal in the aqueous solvent. On the other hand, in the presence of the Na ion alone, *under no known circumstances* will the organism leave an aqueous menstruum for a lipoidal one.

These results assume heightened interest in the light of the very recent work of Raschevsky which on thermodynamic grounds seems to realize the position, viz., that there are experimental conditions where there is not even the necessity of invoking the charge factor in the suspension stability of organic particles.

12. Method of Testing Antiseptics. GEORGE F. REDDISH, Hynson, Westcott and Dunning, Baltimore, Md.

In a previous publication I have taken the privilege of defining antiseptics as follows: "Antiseptics are substances which, when applied to microörganisms, will render them innocuous either by actually killing them or preventing their growth according to the character of the preparation or the method of application." In testing antiseptics, therefore, we must consider the character of the preparation and then the way in which it is to be used in practice. This means that the old phenol coefficient method with *B. typhosus* as the test organism will not be applicable for testing antiseptics except as a means for controlling uniformity of manufacture. The present study has covered many kinds of antiseptic preparations, including ordinary liquid antiseptics, antiseptic ointments, oils, toothpaste, shaving cream, suppositories, antiseptic soaps, gauze, adhesive, catgut, etc. The ordinary test for germicides as we have known it in the past would not be applicable for most of the antiseptic preparations we know today. In many of the prepara-

tions mentioned above a shake agar plate culture of *Staph. aureus* is used and the antiseptic placed on top of the agar. After incubation a clear zone all around the preparation will indicate its activity and the suitability for use in practice. Certain other antiseptics which are not soluble in water are tested by means of small filter paper squares impregnated with test organisms, *Staph. aureus*. Ordinary liquid antiseptics are tested by a method which is a compromise between the Rideal-Walker and the Hygienic Laboratory methods with *Staph. aureus* as the test organism.

13. Comparative Methods for the Testing of Various Germicidal Agents. GEORGE F. LEONARD AND EDNA HEACOCK, E. R. Squibb & Sons, New Brunswick, N. J.

The Hygienic Laboratory Method of determining the phenol coefficient was devised to meet some of the objections and sources of error found in the Rideal-Walker procedure. In the Hygienic Laboratory Method a practical scheme has been devised for the testing of germicidal agents with and without organic matter, giving the phenol coefficient of each. Reddish has recently suggested a modification of the Hygienic Laboratory Method, but we have not been able to obtain as consistent results by the method suggested by him as by the revised Hygienic Laboratory procedure. During the past eight years we have tested a large number of samples of germicidal agents in our Laboratories. The products tested include coal-tar products, silver compounds, mercurial salts and miscellaneous products. We have used various methods of testing and have obtained the most satisfactory results by the use of the Hygienic Laboratory Method controlled by a phenol coefficient test, using a strain of *Staphylococcus aureus*. The *Staphylococcus* phenol coefficient test, as carried out by us, is made similar to the Hygienic Laboratory Method, except that the media is made with a pH of 7.0 and a strain of *Staphylococcus aureus* is used in place of *B. typhosus*. The phenol control and calculations in the *Staphylococcus* test are exactly the same as in the Hygienic Laboratory Method. The results of a number of tests are given showing the consistent results obtained when germicidal agents are tested with 2 organisms belonging to different groups.

14. An Index Useful in Designating the Germicidal Efficiency of Alkaline Washing Compounds. MAX LEVINE AND J. H. BUCHANAN, Department of Bacteriology, Iowa State University, Ames, Iowa.

Alkaline powders are used extensively in bottle and machine cleansing. It is possible to evaluate the germicidal efficiency of these washing com-

pounds on the basis of their sodium hydroxide, sodium phosphate and carbonate content. The germicidal index is the ratio of the weight of a given compound to that of a "standard" compound having the same germicidal efficiency. The methods of determining germicidal indices are detailed.

GENERAL BACTERIOLOGY

Symposium on "Anaerobiosis"

15. An Outline of the History of Studies on Anaerobiosis. Wm. Mansfield Clark, Department of Physiological Chemistry, Johns Hopkins Medical School, Baltimore, Md.

Some of the early observations on "life without oxygen," both in the field of bacteriology and in the field of protozoology are described. The fact of anaerobiosis was early recognized as one of great importance to the isolation of certain species. This alone was often the origin of variety in the technical methods of eliminating oxygen from culture media or of protecting cultures from access to oxygen. These methods when classified roughly and from the point of view of the chemist show frequent duplication of principle. Parallel with the developments in this direction occurred various observations upon the conditions under which anaerobes grow. Various anomalies such as were frequently published under titles like "The growth of an anaerobe in cultures exposed to air" are critically reviewed. The finer points of the observations led to various fine-spun technical terms but at the same time created problems of the greatest delicacy. A few of these are mentioned.

There is presented an analysis of the theoretical relations between equilibrium states in oxidation-reduction processes and partial pressures of oxygen. This analysis indicates how certain aspects of anaerobiosis can be attacked systematically if certain kinetic events in the reduction of dyes by cultures can be quantitatively determined. Various interesting anomalies still unsolved are briefly sketched.

In Bacteriology the subject has been largely one of mere technique. However, it involves matters of the greatest theoretical importance.

16. Reduction Potentials in Bacterial Cultures and Their Relation to Anaerobiosis. Barnett Cohen, Hygienic Laboratory, Washington, D. C.

Bacterial cultures in broth and synthetic media develop progressively

increasing reducing intensities which have been followed electrometrically. Oxidation-reduction indicators, within the limitations imposed by their chemical reactivity and narrow useful range confirm the time: potential curves. The levels of reduction potential attained by cultures of different bacteria are more or less different and characteristic. The pH of the medium affects appreciably the trend of the time:potential curves.

Anaerobes produce a high level of reduction potential and give evidences of the induction of a hydrogen over-voltage. That is, the reduction intensity reaches and then passes above the equilibrium state of hydrogen at atmospheric pressure; and indeed such cultures liberate hydrogen at this stage. Calculation shows that, corresponding to this reduction potential, the pressure of oxygen theoretically in equilibrium with the system under measurement is of the order of 10^{-82} atmosphere.

In a deep semifluid anaerobe culture with surface exposed to air, the reduction potential at different levels is very high except near the surface. In the case of *C. welchii*, the high reduction potential accompanied the turbidity of growth up to about 1 mm. from the surface within twenty-four hours. *Vibrion septique* and *C. edematiens* cultures showed a clear supernatant zone about 1 cm. deep where the reduction potential was not high. This zone was invaded by growth in the next twenty-four hours and the reduction potential was increased simultaneously. Sections of high turbidity (dense growth?) appeared as disks at various levels, but preliminary electrode measurements did not reveal higher reduction potentials in these sections than in the body of the culture.

Oxidation-reduction indicators injected into the ameba under strict anaerobiosis show that the high reduction intensity is present *within the living cell;* and the observations are in agreement with those made on minced tissues and bacterial cultures.

17. *The Principal Postmortem Findings in Guinea Pigs Inoculated with Pure Cultures of Pathogenic Anaerobic Bacilli.* IVAN C. HALL, Department of Bacteriology and Public Health, University of Colorado, Denver, Colo.

The principal pathogenic anaerobic bacilli are *B. tetani*, *B. botulinus*, *B. Welchii*, *B. septicus* (*Vibrion septique*), *B. chauvei*, *B. histolyticus*, *B. Novyi*, *B. Sordellii*, *B. oedematoides*, and *B. hemolyticus*. All of these are obligately anaerobic except *B. histolyticus* which is facultatively aerobic but is included because its general characteristics ally it with the anaerobic bacilli.

The outstanding pathological changes found in guinea pigs inoculated with these organisms or their toxins are shown in the following table:

	OEDEMA	EMPHYSEMA	CONGESTION	HISTOLYSIS
B. tetani	−	−	+	−
B. botulinus	−	−	+	−
B. Welchii	++	+++	+	+
B. septicus	+	++	+++	+
B. chauvei	+	++	+++	+
B. histolyticus	−	−	++	+++
B. Novyi	+++	−	−	−
B. Sordellii	+++	−	+	−
B. oedematoides	+++	−	+	−
B. hemolyticus	++	−	+++	−

The most marked changes occur at the site of inoculation and depend to some extent upon whether the injection is made subcutaneously or intramuscularly, but in the case of the toxins of *B. tetani* amd *B. botulinus* the amount of local congestion is generally very slight while the lungs may show considerably more. There is also a marked hemorrhagic infiltration of the kidney cortex and a striking contraction of the urinary bladder in animals inoculated with cultures of *B. Sordellii* and *B. oedematoides.*

18. The Influence of the Filtrates of Clostridium botulinum and Clostridium sporogenes upon the Growth of Each of these Organisms. C. N. Stark, J. M. Sherman and Pauline W. Stark, Cornell University, Ithaca, N. Y.

Several investigators have noted the absence of toxin in significant amounts when *Clostridium botulinum* is grown in mixed culture with *Clostridium sporogenes.* It has been noted also by Dack that neither of these organisms will grow when inoculated into the unheated bacterial-free filtrate from an aged culture of the other organism. Likewise the growth of either of these organisms is inhibited in its own filtrate.

These observations have been confirmed by us. It has been found that the failure of these organisms to grow satisfactorily in such filtrates is apparently due to the exhaustion of some essential food constituents. For example, if to a sporogenes filtrate some sterile nutrient broth is added, *Clostridium botulinum* is able to make a vigorous growth. Similar results were obtained with *Clostridium sporogenes* in botulinum filtrate, and with each of these organisms in its own filtrate.

19. Anaerobic Bacteria of the Nasopharynx. W. C. NOBLE, JR., AND D. H. BRAINARD, Bacteriological Laboratory, Medical Division, Metropolitan Life Insurance Company, New York City.

Nasopharyngeal cultures have been studied from 18 persons in health and 19 with colds. From 1 subject the culture material was obtained by washing out the nasopharynx with sterile salt solution, from all others by swabbing the nasopharynx. The material was placed in blood agar and cultivated in modified McIntosh and Filde's anaerobic jars. Five species of strict anaerobes were isolated: a Gram-negative coccus, a Gram-positive coccus, and 4 Gram-negative bacilli.

The Gram-negative coccus occurred in all but 5 of the 37 subjects. It was probably *M. gazogenes* of Lewkowicz (*Staph. parvulus* of Veillon and Zuber).

The Gram-positive coccus was found in 2 of the subjects with colds. It formed irregular, confluent zones of hemolysis about groups of colonies on blood agar. We can find no similar organism described in the literature.

Of the 4 species of Gram-negative bacilli, 1 resembled Olitsky and Gates' Group I; the other 3 apparently have not been described before. One of these latter, isolated from a case of acute rhinitis, was long and threadlike. It fermented glucose and levulose slightly. The second, isolated once from a healthy person, varied in morphology. Neither glucose nor levulose was fermented. The colony was small and irregular. The third bacillus, isolated from a case of chronic rhinitis, resembled the second culturally, but its colony was larger, regular and coarsely granular. Morphologically the organism was smaller.

20. Flattened Capillaries for Microscopic Examination of Cultures. W. S. STURGES, Laboratory of the Cudahy Packing Company, Omaha, Nebraska.

Glass tubing is flattened and drawn out into minute ribbon shaped capillaries having excellent optical properties not possessed by cylindrical tubes. Filled with drops of broth cultures and sealed off, these constitute very convenient cells for the microscopic study of motility, morphology, growth, etc. The capiliaries are either filled with the broth culture after growth has taken place or immersed in tubes of media before sterilization and removed for examination after appropriate cultivation of the organism of these tubes. This technique has been found especially valuable in examining anaerobes for motility, probably because of the exclusion of oxygen.

21. Motility of C. bifermentans. W. S. Sturges and E. T. Drake, Laboratory of the Cudahy Packing Company, Omaha, Nebraska.

Fourteen strains of *C. bifermentans* and *C. centrosporogenes*, including 4 authentic *Bifermentans* strains from I. C. Hall, were studied in flattened capillaries. By this method active motility was easily demonstrated in young cultures of all strains. By other methods *Centrosporogenes* was usually motile and *Bifermentans* not. This is true of young cultures in both broth and meat. Excellent flagella stains have been obtained showing peritrichous flagella on both organisms. Considerable uncertainty, therefore, attaches to the main point of distinction between *Centrosporogenes* and *Bifermentans*.

AGRICULTURAL AND INDUSTRIAL BACTERIOLOGY

1. The Identification of B. radiobacter and its Occurrence in Soil. Nathan R. Smith, U. S. Department of Agriculture, Washington, D. C.

The occurrence of *B. radiobacter* in soil may be determined by the following technique: dilutions of the soil, 1:10,000 or greater depending upon the soil, are prepared in steps of 10×, transferring 10 to 90 cc. of tap water after the first dilution which may be varied, but never greater than 1:50. To the proper dilution, for radiobacter 0.5 cc. of a 0.1 per cent crystal violet is added and 1 cc. plated within two or three minutes on a glycerine-nitrate agar of the following constitution: Soil extract (1 part soil, 2 parts water) 1000 cc., dipotassium phosphate 1 gram, sodium nitrate 1 gram, glycerine 10 grams and agar 10 grams. Reaction should be adjusted to pH 7.0 if it is below this. The crystal violet inhibits the growth of *Actinomyces* and Gram-positive bacteria but does not interfere with the development of Gram-negative bacteria. The colonies of radiobacter are very characteristic. They are raised, smooth, glistening, center opaque and edge transparent. After a little experience with this medium, radiobacter can be recognized in 80 per cent of the cases. In studying hundreds of pure cultures of radiobacter, great variation in their physiology has been noted. The browning of milk cultures is the most reliable but there are radiobacters which do not do this constantly. Other characteristics are even more variable.

The growth of legumes increases the numbers of *B. radiobacter* in soil beginning with the third week and extending some weeks after harvesting of the crop. If cereals are planted at once, the numbers gradually become less. These results are easily demonstrated in greenhouse work.

In field work, radiobacter is found in greatest numbers near the plant, often none being found one foot distant from the plant. Cowpeas, field peas, vetch and soybeans stimulate radiobacter in the order named which coincides with the order of their beneficial effect on soil.

2. Fatty Acids as Sources of Energy for Azotobactor. P. L. GAINEY, Kansas Agricultural Experiment Station, Manhattan, Kan. (With exhibit.)

The study involved a comparison of the rapidity of growth, change in reaction, nitrogen fixed, and acid consumed when calcium salts of the lower fatty acids were supplied as the only source of organic matter in liquid cultures of *Azotobacter chroococcum* and other strains. The various strains of *Azotobacter* behave quite differently with respect to their ability to utilize different fatty acids, some cultures being very limited in this respect while others can assimilate a wide variety of acids. Iso-compounds are less easily metabolized than are the normal acids. There is a marked reduction in the hydrogen-ion concentration in a medium in which an acid is utilized to any appreciable extent. The quantity of nitrogen fixed is more or less proportional to the quantity of acid consumed. The quantity of nitrogen fixed per unit weight of acid consumed increases as the molecular weight increases, provided comparisons are limited either to normal or iso-acids. This apparently holds true even if the comparisons are based upon the energy content of the acid consumed, indicating a more efficient utilization of acids of higher molecular weight. The cation with which the acid is combined plays a very important rôle in determining the ability of an organism to utilize the acid.

3. The Relation Between the Legume Root Nodule Bacteria and the Host Plant With Reference to Plant Immunity. D. H. DUNHAM AND I. L. BALDWIN, University of Wisconsin, Madison, Wis.

An attempt has been made to determine the effect of nodule formation upon the host plant. Certain investigators have claimed that an immunity is set up in the plant by nodule production while others feel that the nitrogen supply of the plant is the controlling factor in determining the production of additional nodules.

Among the root nodule bacteria of alfalfa, red clover and soybeans, 2 broad general subgroups have been established, differing in cultural characteristics, physiological properties and serological reactions. Of the 2 strains chosen for use in each cross inoculation group, one possessed

the power of bringing about high fixation of nitrogen and the other a low fixation of nitrogen. Strain identification was based upon cultural characteristics, fermentation reactions, sensitiveness to acidity, and also agglutination tests.

Experiments with alfalfa, red clover and soybeans have shown that a single plant may bear at the same time nodules, part of which have been induced by one strain of bacteria and part by another. In no case, however, has a single nodule been discovered containing more than one strain of the root nodule bacteria.

4. Studies on the Composition of the Cells and Gum of the Root Nodule Bacteria. E. W. Hopkins, E. B. Fred and W. H. Peterson, University of Wisconsin, Madison, Wis.

The bacteria were grown on yeast-water-mannitol medium from which the cells were removed by centrifugation and the gum by precipitation with acetone. The gum was purified by reprecipitation. The dried samples were analyzed for carbon and nitrogen with the following results calculated on the ash free basis: Cells, C. 53.8 per cent, N. 4.69 per cent; Gum, C. 38.8 per cent, N. 0.0 per cent. Glucose was obtained from the gum by acid hydrolysis.

5. The Effect of Fertilizers on Soybeans. Harold W. Batchelor and Irvin H. Curie, Ohio Agricultural Experiment Station, Wooster, Ohio.

A farm grain drill, of the shovel type with fertilizer attachment, was rebuilt so that 5 rows of seed were drilled at one time, the fertilizer being placed in 4 different positions with respect to the seed: 4 inches at the side of the seed, with the seed, 1 inch below the seed, and on the surface of the soil, above the seed. The fifth row was left unfertilized as a control.

Three fertilizers, 0–16–0, 0–14–4, and 0–10–10 were each used at 4 different rates: 100, 200, 300, and 400 pounds per acre. In all, 60 rows, 3½ rods long were used with 3 additional rows of sterilized seed as checks on field sterility. Manchu seed that had been inoculated with a pure culture was used. The studies were conducted on Wooster Silt Loam in 1926 and on Canfield Silt Loam in 1927. These soils had not previously been seeded to soybeans nor fertilized within eight years. Germination of seed, green and dry weight of nodules from 25 plants, percentage of plants inoculated, dry weight and nitrogen content of entire plants, and weight of seed from one-rod rows were determined.

The fertilizers gave marked increases over the control rows, in the weight of nodules, the percentage of plants inoculated, the weight and total nitrogen content of plants, and the weight of seed produced. Fertilizers placed at the side, with, and below the seed, in general, gave greater increases than those placed above the seed.

The 0–16–0, 0–14–4, and 0–10–10 gave respectively increasing weights of nodules and nitrogen content of plants. The 0–16–0, however, gave consistently greater weight of seed than the other fertilizers. The different fertilizers gave no appreciable differences in weight of plants.

Toxicity of the fertilizers was not evident except when placed with the seed. When so placed, the higher additions reduced the germination to 30 per cent as compared with the controls. Toxicity was evidenced in increasing amounts in 0–16–0, 0–14–4, and 0–10–10 respectively.

The experiments indicate that the proper placing of a sufficient amount of one of these fertilizers may appreciably increase the inoculation and yield of soybeans on some soils.

6. The Effect of Freezing on Yeasts. F. W. Tanner and Beatrice Williamson, Department of Bacteriology, University of Illinois, Urbana, Ill.

Little study seems to have been given this question for there are few reports of investigations in the literature. The several which have been found were carried on over a short period of a few hours to a day or so. The data reported here were secured with 8 species of budding fungi and 4 species of bacteria. The latter were included since so much more work has been done with them; they functioned, then, as controls. The organisms were suspended in broth and physiological sodium chloride. About 1.5 cc. of these suspensions were placed in 2 cc. sterile ampoules which were sealed and placed in the trays of an electric refrigerator. The observations there stopped on the bacteria after 80 weeks and on some of the yeasts after 106 weeks. There was a steady decrease in the number of viable cells of all organisms. The rate of death in all cases seemed to follow the logarithmic law of death. A noticeable difference in resistance to prolonged freezing among the species used was noticed. *Saccharomyces cerevisiae*, for instance, seemed to be more resistant than the other species. *Pichia membranaefaciens* was least resistant.

7. Should Bacterial Standards for Swimming Pools Be Revised? Jean Broadhurst and Anne Dix, Teachers College, Columbia University, New York City. (Exhibit only.)

The high resistance of *Alkaligines fecalis* to chlorine may cause high

bacterial counts even when swimming pools are treated with hypochlorites in much greater concentrations than usually recommended. In experimental work (liter quantities) *Alkaligines fecalis* survives 5 parts per million of chlorine (pH 7.53), and is cut out only when the chlorine is as great as 6 parts per million. There is indication that this organism persists in rapid sand filters, and causes practically pure cultures on examination plates reaching tens of thousands per cubic centimeters even when *coli* is lacking in 1-cc. amounts. Since *coli* is eliminated at 1 to 1½ p.p.m., can we assume a negative presumptive test for *coli* indicates safety, even though the actual bacterial count is much in excess of the 300 limit set at present?

8. Is the Statistical Method Applicable to the Bacterial Plate Count? C. S. Mudge and B. M. Lawler, Division of Diary Industry, University of California, College of Agriculture, Davis, California.

Nine samples of milk were plated using standard procedure in series of 75. Statistical constants were determined from the averages obtained. Seven of the 9 samples were found to have coefficients of variation of from 50 to 134 per cent. Since the plates were numbered in the order of their making, information was obtained which showed that the plates poured at the end of any one experiment gave a higher count than those first poured. Coefficients of variation were determined for the first 40 plates which showed a marked decrease over that for the total 75. It is suggested that due to the conditions encountered in the operation of plating, the statistical constants are of little value.

9. Anomalous Bacterial Plate Counts from Milk. Harry A. Harding and Archibald R. Ward, Dairy Research Division, Mathews Industries, Inc., Detroit, Mich.

An early example of puzzling results of bacterial plate counts is that recorded by B. R. Rickards, in which he comments that the "pasteurizer was evidently not being run successfully, the pasteurizer acting not as such but as an incubator." His counts are: before pasteurizing, 716,000; after pasteurizing, 2,196,000. Increase 200 per cent approximately.

An experiment by us in coöperation with F. O. Adams of the Detroit Health Department in which we pasteurized milk for thirty minutes at 143° to 145°F. in test tubes, yielded the following results along with

others where normal reduction in count occurred: Number of bacteria per cubic centimeter in samples of raw milk, 65,000, 118,000, 3,500,000, 3,800,000, 3,200,000; Number of bacteria per cubic centimeter in same samples of milk after pasteurization, 5,000,000, 5,000,000, 5,000,000, 4,500,000, 3,000,000.

These results show extraordinary increase in the count of pasteurized milk over the raw count in 2 instances and in 3 instances no appreciable reduction in count by pasteurization.

Unexpected high counts of pasteurized high grade raw milk and of certified milk were obtained in another series. The pasteurization was carried out in test tubes for thirty minutes at 145°F. The counts of the raw milk were made by both standard plates and Frost little plates, while the counts of the pasteurized milk were made by the latter method.

The foregoing examples are presented merely to point out the existence of a problem, for information to explain them adequately is not available. Enough progress has been made in the study of the problem to indicate the importance of the influence on counts of thermophilic and heat resistant flora as well as the influence of previous refrigeration of milk in preventing growth on plates.

10. Some Factors that May Affect the Plate Count of Milk. WILLIAM R. ALBUS, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

Data are presented showing that lack of due consideration of the principles of bacterial growth may be responsible for the reported inaccuracies in the plate counts of milk. It has been found possible to make plate counts of milk that check closely, when factors affecting bacterial growth are controlled.

11. The Effect of Alkali Solutions Upon the Bacteria Normally Found in Unwashed Milk Bottles. C. S. MUDGE AND B. M. LAWLER, Division of Dairy Industry, University of California, College of Agriculture, Davis, California.

The wash water of 40 extremely dirty bottles returned to a milk plant was submitted to the action of alkalies of various strengths, for various times and temperatures. From these results smooth curves have been plotted for the action of these alkalies under different conditions making it possible to predict the probable lethal factors for the alkalies.

12. The Inhibiting Effect of St. lactis on Lactobacillus bulgaricus. L. A. ROGERS, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

If milk is inoculated simultaneously with *St. lactis* and *L. bulgaricus* both will grow. If the *St. lactis* inoculation is much heavier than the bulgaricus inoculation, or if the streptococcus is allowed to develop for a few hours before the bulgaricus inoculation, there is little or no development of bulgaricus. After a few days the bulgaricus begins to develop. The inhibiting effect is removed by heating or by filtering through clay or plaster of paris filters. The inhibiting action is independent of the reaction of the culture.

13. Oxidation-Reduction Potentials in Milk. H. R. THORNTON AND E. G. HASTINGS, Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wis.

The positive E_h limits of all the milks studied lay between +0.2 volts and +0.27 volts. The negative potential limits uniformly approximated E_h −0.2 volts. It is suggested that this uniformity of negative limits was due to the predominating influence of *Streptococcus lactis* in the milk.

The reduction of methylene blue in milk took place over a zone somewhat more positive than the theoretical for this dye at this pH. This was probably due to the influence of another oxidation-reduction system or systems in the milk. A slight but inconsequential "poising" effect due to the methylene blue was observed.

The effect of oxygen upon oxidation-reduction potentials and upon the reduction of methylene blue points to the conclusion that the reduction of this dye in milk may not indicate absence of oxygen. Comparative reduction times for 3 indicators show that as a test of quality in milk the use of a dye reducing at a more positive potential than that at which the reduction of methylene blue takes place is unsound. A faint blue color frequently persists in the milk for some time after the bulk of the methylene blue has unquestionably reduced. It is suggested that this is due to an impurity in the dye which reduces at a potential more negative than does methylene blue.

14. Some Non-Spore-Forming Thermoduric Bacteria from Pasteurized Milk. A. H. ROBERTSON, Vermont Agricultural Experiment Station, Burlington, Vt.

A series of pasteurized milk plates from various cities in this county

were sent to the New York Agricultural Experiment Station for the purpose of studying the new so-called "pin-point" flora of pasteurized milk. Nearly 200 cultures were isolated from these plates. Enrichment methods were employed to isolate another series of non-spore-forming species which survive pasteurization. A further selection of these cultures for future study consisted of determining those cultures which gave over 90 per cent survival as determined by the agar plate method.

The cultures possessing the greatest resistance to heat are members of five species, as follows: *Microbacterium lacticum* Orla-Jensen (1919), *Streptococcus thermophilus* Orla-Jensen (1919), *Sarcina lutea* Schröter (1872), *Micrococcus conglomeratus* Migula (1900) and *Sarcina rosea* Schröter (1872). *Microbacterium lacticum* has been developed by enrichment methods from 5 out of 60 milk samples from individual producers. *Streptococcus thermophilus* is the only species which grows rapidly at 25° to 37.5°C. The presence of the above species in market milk as determined by fermentation studies can not be regarded as dangerous to health.

15. Influence of Age of Culture on the Heat Resistance of Non-Spore-Forming Bacteria. A. H. Robertson, Vermont Agricultural Experiment Station, Burlington, Vt.

A study of the heat resistance of milk cultures of *Microbacterium lacticum*, Orla-Jensen, *Sarcina lutea* Schröter and *Streptococcus thermophilus*, Orla-Jensen has been completed. These species are the three most heat-resistant non-spore-forming types isolated. Cultures were pasteurized for thirty minutes at several different temperatures, 62.8°C. being the temperature most frequently used. Sub-samples of the original culture were pasteurized immediately after inoculation and at such times thereafter as desired. Plate counts were made both before and after pasteurization in order to determine the numbers killed.

The young, rapidly growing or adolescent cells are more susceptible than the older or mature cells to the killing action of high temperatures. As the temperatures used approach the thermal death points of the organisms, the degree of killing at any one age is greater and extends over a longer period of time. Most efficient pasteurization of milk can be secured if the heat is applied before the microörganisms have passed the accelerative growth stage.

16. Thermophilic Organisms in Milk. P. S. PRICKETT, Agricultural Experiment Station, Geneva, N. Y.

During recent years in the sanitary control of milk much attention has been given to "pin-point" colonies and their causal organisms—thermoduric (heat-resistant but not thermophilic) and facultative thermophilic bacteria. That only such organisms would attract attention in the sanitary control of market milk is to be expected as all incubation in routine control laboratories is done at 37°C. Such methods give no information concerning the obligate thermophilic flora and its economic importance such as is obtained by the use of the microscope and incubation of plates at 56° and 65°C.

Several large market milk pasteurizing plants using continuous flow type pasteurizers that are in operation for a long period each day report that the milk shows an increase in acidity towards the end of the run. This is quite important from the standpoint of the keeping quality of the milk. Certain large milk powder plants also report an increase in acidity in skim milk stored for some hours in their hot wells and holding tanks.

Studies in a large plant using a continuous flow type pasteurizer that is in operation for five to seven hours daily revealed the presence of obligate thermophiles. Microscopic examination showed the predominant flora of the milk towards the end of the run to be large rods among which spores were frequent. Among other characteristics these organisms curdled and digested milk at pasteurizing temperatures. Microscopic examination showed the foam to contain large numbers of large rods. A spore-forming, facultative thermophilic rod which produced a rennet-like curd in milk followed by digestion was isolated from the foam.

Studies on skim milk from the hot wells of a milk powder plant disclosed at least two types of spore-forming thermophiles (probably facultative) to be present. These organisms in pure culture were able to grow abundantly in milk incubated at 56°C., and one type caused a distinct increase in the acidity of the milk.

17. A Study of the Cocci Resisting Pasteurization Temperatures. G. J. HUCKER, Agricultural Experiment Station, Geneva, N. Y.

A study of 82 strains of cocci resisting pasteurization indicated that *S. thermophilus*, Orla-Jensen, is the predominating coccus in pasteurized milk. Of the other lactic acid streptococci *S. faecium*, Orla-Jensen, and *S. liquefaciens*, Orla-Jensen, were found, both of which, in many instances, are able to resist the usual pasteurization temperatures.

Of the total number of strains studied, 31 proved to be micrococci, although no particular species predominated. *M. epidermidus* (Kligler), Hucker, *M. candidus*, Cohn, *M. varians*, Migula, and *M. luteus* (Schroeter), Cohn, were the most commonly occurring micrococci.

It was also noted that pasteurized milk which had been held at 20°C. to 30°C. for four hours previous to pasteurization may contain relatively large numbers of *S. thermophilus* while the same milk if held at 10°C. for the same length of time before pasteurization had a tendency to yield a miscellaneous flora when pasteurized. Milk which had been held at 10°C. for any length of time previous to pasteurization rarely yielded *S. thermophilus*.

18. Studies on the Proteolytic Bacteria of Milk. W. C. Frazier and Philip Rupp, Bureau of Dairy Industry, U. S. Department of Agriculture, Washington, D. C.

A study was made on 226 aerobic or facultative, mesophilic organisms isolated from milk. The organisms were divided into species as far as possible and were also divided in a general way on their action on milk, casein and gelatin. Of the 133 cocci, 11 species (126 organisms) were lactose fermenting and 2 species (7 organisms) were not. Of the 93 rods, 2 species (35 organisms) were lactose fermenting and 18 species (58 organisms) were not. Of the lactose fermenting cocci 4 species (30 organisms) were proteolytic in milk but not in casein solutions and only slightly proteolytic in gelatin solution, while 7 species (96 organisms) were proteolytic in milk and in casein and gelatin solutions.

The proteolytic spore-forming rods fall into 3 main groups on the basis of their action on milk: (1) The high acid, low amino-nitrogen group. These are lactose fermenting organisms and produce a comparatively small increase in amino-nitrogen in milk within ten days. *B. albolactis* is an example. (2) The low acid, low amino-nitrogen group. This group produces a slightly acid reaction and a comparatively small increase in amino-nitrogen. *B. mesentericus* and *B. simplex* are examples. (3) The low acid, high amino-nitrogen group. This group produces an alkaline reaction and a considerable increase in amino nitrogen. *B. cereus* and *B. vulgatus* are examples.

19. The Micrococci Present in the Normal Cow's Udder. Alice F. Breed, Agricultural Experiment Station, Geneva, N. Y.

In the course of the present study, 176 cultures of micrococci were isolated at random from the udders of cows in the Station herd and their

identity traced by the key devised by Hucker (1924). They have been found to belong to at least 12 species which are named in order of their relative abundance as follows: *M. aureus, M. aurantiacus, M. freudenreichii, M. albus, M. candidus, M. epidermidus, M. varians, M. citreus, M. flavus, M. conglomeratus, M. luteus, M. casei.* Five remaining cultures were reddish or pink chromogens that did not agree exactly in characters with any of the red cocci described by Hucker.

Chromogenesis was somewhat variable especially among the orange species.

The average germ content of the milk from the udders of the cows examined was in excess of 500 per cubic centimeter, the figure found by Harding and Wilson (1913).

20. The Distribution of Bacteria in Butter. Otto Rahn and H. H. Boysen, Department of Dairy Industry, Cornell University, Ithaca, N. Y.

The junior author has shown that butter contains ordinarily between 12 and 18 billion moisture droplets per gram; of this number, the vast majority amounting to about 7 per cent of the moisture in butter are smaller than 15μ; about 2 per cent of the moisture in butter is found in droplets between 15 and 100μ diameter, and the rest of the moisture, about 4 per cent, is contained in droplets larger than 100μ.

The number of bacteria in butter is much smaller than the number of moisture droplets, consequently, there must be many droplets free from bacteria, and as there is practically no connection between the droplets, they remain sterile and unchanged. A computation shows that with 30,000 bacteria per gram of fresh butter, the droplets under 100μ diameter are hardly infected. If fresh butter contains 15,000,000 cells per gram, however, practically all droplets over 10μ and also a large number of the smaller ones contain bacteria, and less than 3 per cent of the moisture in butter remains sterile.

It was possible to separate the larger droplets from the smaller ones by centrifuging at different temperatures. But the number of bacteria was smaller in the larger droplets on account of adsorption. Even in butter a few hours after churning when we should expect a perfectly uniform distribution of bacteria, the small droplets contained about 10 times as many bacteria as the first centrifugate representing the larger droplets. This ratio decreased as the butter became older.

In order to get results uninfluenced by adsorption and to prove the more complete deterioration in the larger droplets in spite of the

apparent contradiction of the plate counts, the hydrogen ion concentration was determined. In a seven-day-old butter, not washed, the first centrifugate representing the largest moisture droplets proved to be much more acid than the rest of the moisture in butter, the pH being 4.65 as compared with 5.45 in the rest of the moisture.

The keeping quality of the butter should be the greater the finer the distribution of moisture. A butter which is overworked should undergo less bacterial deterioration than a butter with many large droplets. These computations will not hold for molds which have the power to grow from one droplet to the other, nor for some yeasts which form mycelial structures.

21. The Thermophilic Flora of Sugar in its Relation to Canning. E. J. CAMERON AND C. C. WILLIAMS, National Canners Association Research Laboratories, Washington, D. C.

There are 3 groups of thermophilic spore bearing bacteria of major importance in the spoilage of nonacid canned vegetables. These are (1) the "flat sour" type, producing acid but no gas; (2) thermophilic anaerobes, forming acid and gas other than H_2S; (3) thermopholic anaerobes, distinct from the second group in that there is considerable H_2S production.

During the canning season of 1926 it was established that sugar was a vehicle of major importance in bringing thermophilic contamination to the canning plant. Spores furnished by the sugar served to inoculate susceptible points in the canning operation where, under faulty conditions of control, the bacteria thus supplied were able to increase.

Spores of "flat sour" type thermophiles were found in essentially all of the cane sugar samples examined. In the refined product counts as high as 100 per gram were obtained. Anaerobic thermophiles (no H_2S) were encountered periodically. Anaerobic thermophiles (H_2S) were found rarely but notably in one instance from a sample received from a canning which had suffered loss through spoilage by these bacteria.

"Flat sour" spores were not often found in white beet sugar but this appeared to contain spores of thermophilic anaerobes (no H_2S) in larger numbers than in cane sugar. There were no positive tests for H_2S-producing thermophilic anaerobes.

A study of a sugar refining operation was made from the standpoint of the prevalence of "flat sour" type thermophiles. There appeared to be three bacteriological phases in the refining process. Contamination entered with the raw sugar and persisted up to the Sweetland Press. A

condition approaching sterility was evident after the Sweetland Press and clear filter operations. The third phase was one of highest contamination and existed in the syrup storage tanks and vacuum pans. Syrup counts as high as 1000 spores per gram of sugar were obtained from the pans.

22. Distribution of Thermophilic Spoilage Bacteria. L. H. JAMES, Bureau of Chemistry and Soils, Washington, D. C.

The National Canners Association have shown the existence of thermophilic bacteria whose spores are capable of surviving the ordinary commercial process given to canned foods, and which may subsequently produce "flat sour" or "hard swell" types of decomposition. In more recent studies they have isolated another resistant anaerobe whose characteristic feature is a marked production of hydrogen sulfide. The presence of such organisms markedly increases the danger of wholesale spoilage of canned foods and a study of their natural habitats in and around two commercial canneries was made.

Cannery 1. (Food—sweet corn.) No excessive spoilage had been encountered at this plant. Samples of corn representing various stages in the factory's handling of the product revealed no organisms of the "flat sour" type, a few of the "sulfur" type and a predominance of those producing hard swells. The finished product (following efficient cooking and cooling) was sterile.

Four samples of the sugar used in making the syrup gave the following results: all 4 contained the "flat sour" type, 2 contained the "sulfur" type, and 1 contained the "hard swell" type of thermophiles. Four samples of salt, also used in making the syrup, revealed no spoilage organisms, the high concentration of salt used, however, making the results inconclusive. Of 9 samples of soil collected in fertilized fields, 89 per cent contained the "hard swell" organism, 66 per cent harbored the "sulfur" type and none of them revealed any organism of the "flat sour" type.

The spoilage organisms in 3 samples of manure were found to be as follows: the "hard swell" and "sulfur" types were present in all of the samples, while the "flat sour" type was not found in any of them.

Cannery 2. (Food—peas.) This cannery had not experienced excessive spoilage. Examination of line samples revealed an abundance of the "flat sour" type, but failed to show any of the "sulfur" or "hard swell" types. The greatest number of the "flat sour" type were found in peas immediately following their treatment in an accessory blanching tank. Cans of the product which had been efficiently cooked harbored

living spores of this type. Relining the blanching tank with metal partially removed the difficulty.

23. The China Blue-Aurin-Cellulose Medium for the Physiological Investigation of Cellulose Fermentation. J. R. SANBORN, Department of Bacteriology, Macdonald College, P. Que., Canada.

Raw cotton and the seed fibers of the milkweed proved to be suitable forms of cellulose for fermentation studies. Besides the precipitated raw cotton used in the preparation of the China blue-aurin-cellulose medium, a basic nutrient solution was employed consisting of 1 gram each of potassium phosphate (dibasic), magnesium sulphate, sodium carbonate, and ammonium sulphate, dissolved in a liter of distilled water. With the addition of the China blue-aurin indicator the medium becomes of a bright red color. Both the liquid and agar forms of the medium were employed successfully in the isolation and physiological investigation of various species of cellulose-decomposing bacteria and filamentous fungi.

In the more active fermentations a clear blue zone appeared about the growth in plate culture in twenty-four to forty-eight hours, the blue color extending well into the red medium. As a rule, the colonies of filamentous fungi revealed the China blue-aurin reaction for longer periods than in the case of the bacterial growth, though the clear zones were usually plainly visible even after the fading of the blue. Among the active cellulose-destroying fungi studied were members of the genera *Fusarium*, *Penicillium*, *Acrostalagmus*, and *Chaetomium*.

24. Pectin Fermenting Bacteria. L. A. BURKEY. Department of Bacteriology, Iowa State University, Ames, Iowa.

Organisms capable of fermenting pectin with production of acid or of acid and gas have been isolated from a considerable number of sources. The characteristics of most of the species and strains studied place them quite definitely in the group containing *Aerobacter* (*Bacterium*) *aerogenes*. Descriptions and differential characters of new species are enumerated. The significance of certain of these forms, particularly in retting processes, is discussed.

25. The Bactericidal Action of Smoke (as Used in the Smoke-Curing of Fish). ERNEST HESS, Biological Board of Canada, Halifax, Canada.

A short account of the principles and methods of smoking fish (finnan haddies and smoked fillets) is given.

Artificial media were used instead of fish flesh on account of the difficulties the latter presents in quantitative work (especially the smoked product), and due to the impossibility of sterilizing fish flesh without changing its character entirely and to obtain a uniform culture material. Three strains of bacteria (a staphylococcus, a non-spore-forming rod (*Prot. vulgaris*) and a spore former of the *Subtilis-mesentericus* group) were used in several series of experiments to determine the bactericidal action of smoke under various conditions. The arbitrary scale: "Cubic foot of air admitted to the smoke producer per hour" gave a fair indication of the smoke concentration. An increase of the amount of air admitted per hour caused uniformly a greater reduction of bacteria, i.e., an increase of the bactericidal power of the smoke, as well as visibly, a higher density or opaqueness of the smoke. Smoke from different sources (sawdust from hardwood, softwood and mixtures of both) showed practically no difference in bactericidal power after six hours' action upon the test organisms. The rate of reduction of non-spore-formers under experimental conditions was greatest between one-half to two hours of exposure to fairly weak smoke, with an initial weakening of the bacteria during the first half hour and the killing of the more resistant forms during the third hour of exposure. The more concentrated the smoke, the greater was the initial and the total rates of reduction. Bacterial spores were found to be very resistant against smoke, the resistance increasing with increasing age of the spore cultures; 71 per cent of the spores from a seven months old spore culture survived exposure to a dense smoke for seven hours, while non-sporing organisms were killed in one to two hours by the same smoke. Penetration of smoke was found to be fairly rapid through peptone-water, slow through a flour paste and very irregular through slices of fish muscle. The more concentrated the smoke the higher its penetrating power. Smoke constituents which had been absorbed by the smoked media during the exposure, continued to exert a bactericidal action upon the test organisms after the smoking process was finished. Formaldehyde vapors, containing 3 or more grams of formaldehyde per 100 cubic feet, approximately in the same concentration as they occur in ordinary smoke, have a decided bactericidal action. Formaldehyde is considered one of the chief bactericidal constituents of smoke. The resistance of bacteria against smoke is greatest in media of optimum salt concentration. The influence of the presence of proteins upon the bactericidal action of smoke was determined, using peptone solutions and fish extracts. Both were shown to have a

decreasing—"quenching" action upon the effect of smoking, this was less conspicuous in case of dense smoke than when less dense smoke was used. The hydrogen-ion concentration of unbuffered media is greatly increased during smoking. The bactericidal action of smoke in buffered media was shown to be greatest in an acid medium, and decreasing constantly towards the alkaline side of neutrality.

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Symposium on "Bacterial Toxins"

1. The Nature of Diphtheria Toxin. Arthur Locke and E. R. Main, St. Luke's Hospital, Chicago, Ill.

The paper discusses: (1) the conditions which favor the production of toxin in cultures of toxicogenic diphtheria bacilli; (2) the properties and reactions of the toxin, *in vitro;* (3) the immunologic and histologic changes which follow its injection into animals; (4) the properties which diphtheria toxin shares in common with other toxins and with antigens in general; and (5) the conclusions which may be drawn as to the nature of diphtheria toxin. The principal new data presented relate to the mechanism of toxin production, the preparation and properties of a highly purified toxin, and the source of the latter's toxicity.

2. Destruction of Botulinum Toxin by Milk Bacteria. J. M. Sherman, C. N. Stark and Pauline W. Stark, Cornell University, Ithaca, N. Y.

The unique freedom of milk and milk products as agents in the dissemination of botulism is of great interest. Although fresh milk may ordinarily be used before bacterial growth has been sufficiently extensive to be dangerous from the standpoint of botulinum poisoning, such is not at all the case with cheese and certain other milk products. Indeed, from the standpoint of chemical composition, cheese would appear to be an excellent medium for the production of this toxin. The explanation which might appear valid is that the acidity of most types of cheese is sufficiently high to inhibit the growth of *Clostridium botulinum*, but such an explanation would not hold for all types of cheese, and from some of the experimental results which have been published concerning the acid limits for growth of this organism, would indicate that a complete explanation cannot be founded entirely on the basis of acidity in the case of any type of cheese. From our results on the destruction of toxins by bacteria it appeared plausible that some of the characteristic

milk organisms might have the power of destroying the toxin produced by *Clostridium botulinum*.

It has been found that *Streptococcus lactis* and *Lactobacillus casei* have the power to destroy botulinum toxin. This is of particular interest in connection with cheese, since in most types of cheese *Streptococcus lactis* is the predominating organism during the early stages of ripening, while *Lactobacillus casei* is one of the predominating organisms in all types of cheese during the later stages of ripening.

3. *The Action of Haematoxins upon Oxygenated and Reduced Blood Cells*, GUILFORD B. REED AND J. H. ORR, Queen's University, Kingston. Ontario, Canada.

In agreement with the work of Neill, it was found that the oxidation of *B. welchii* toxin by the exposure of culture filtrates to the air resulted in loss of haematoxic action. The degree of oxygenation of the red cells with which the haematoxin is combined proves to have a similar influence upon the reaction. When *B. welchii* toxin is mixed with standard emulsions of red cells which have been freshly oxygenated or reduced with CO_2, 2 to 3 times more toxin is required to produce a given amount of haemolysis in the oxygenated than in the reduced blood. Quantitative data of the influence of the degree of oxygenation is included.

4. *A Study of the Reactions Between Toxins and Antisera Produced with Hemolytic Streptococci.* MARY B. KIRKBRIDE, MARY W. WHEELER AND JESSIE L. HENDRY, Division of Laboratories and Research, State Department of Health, Albany, N. Y.

This paper presents the results of comparative tests of the neutralization of monovalent antistreptococcus sera with the homologous and heterológous toxins as determined by intracutaneous tests upon persons and goats.

5. *A Gastro-Intestinal Poison Produced by Bacterium Enteritidis and by Bacterium Paratyphosum B (Aertrycke Type).* SARA E. BRANHAM, LUCILLE ROBEY AND LOIS A. DAY, Department of Hygiene and Bacteriology, The University of Chicago, Chicago, Illinois.

This study was made with 17 strains of *Bacterium enteritidis* and *Bacterium paratyphosum B* (Aertrycke type) which were isolated from

cases of food poisoning. All of these strains produced typical fatal infection in mice, when given by mouth. The organisms could regularly be recovered and identified.

Series of mice were fed by catheter: (1) heat-killed broth cultures, (2) autolyzed suspensions, and (3) sterile Berkefeld filtrates of broth cultures. The highest mortality, 41 per cent, occurred in the group fed with filtrates. No paratyphoid organisms could be recovered from these. The autolyzed suspensions had very little effect.

Curves were plotted showing the incidence of mortality, in these groups of mice, including the group fed with the living cultures. These showed that the greatest number of deaths among the mice fed with living organisms occurred on the fifth and sixth days after feeding, whereas in the group fed boiled broth cultures and filtrates, the highest death rate occurred between the tenth and fourteenth days. Mice injected intraperitoneally with these latter materials died in two to four days. Failure to recover paratyphoid bacteria from these latter groups suggested the presence in the filtrates and broth cultures of a toxic substance whose action is usually masked by the quicker course of infection with the living organisms.

Therefore, more than 300 mice were fed filtrates of cultures of various ages in different kinds of media. Constantly, a certain proportion of these mice died between the tenth and fourteenth days. They presented a uniform pathological picture at autopsy. No paratyphoid organisms could be recovered from them. Deaths did not occur in any series of mice kept under identical conditions, but fed with the uninoculated media.

Filtrates from cultures in different kinds of media varied greatly in their poison content: some lots of beef heart medium were associated with a mortality as high as 80 per cent; veal infusion broth was usually followed by the death of 20 to 40 per cent; some were uniformly nontoxic. Filtrates from twenty-four-hour cultures seemed most potent. The poison was not injured by autoclaving at 15 pounds for fifteen minutes.

There seems, undoubtedly, to be a heat resistant poison in young cultures of *Bacterium enteritidis* and *Bacterium paratyphosum B* (Aertrycke type) which is toxic for mice when taken by mouth. This poison seems to be different from the toxic principle previously described, which acts only on intravenous injection. Whether it is a bacterial toxin or is a poison formed by the action of the microorganisms on the medium, can be determined only by further investigation.

6. Properties of a Toxin Produced by an Organism Isolated in Periodic Ophthalmia in the Horse. EDWARD C. ROSENOW, Division of Experimental Bacteriology, The Mayo Foundation, Rochester, Minnesota.

The incidence of the isolation of the organism in periodic ophthalmia and from feeds is discussed. The effect on injection in animals of filtrates containing the toxin, especially the specific affinity for the eye, heat resistance, solubility in ether, behavior towards adsorbing agents, and the preparation of an antitoxic serum in the horse are described.

7. Simplified Cataphoresis Methods to Estimate Diphtheria Bacillus Virulence. I. S. FALK, L. B. JENSEN AND J. H. MILLS, University of Chicago, Chicago, Ill.

Demonstration of apparatus.

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8. Studies on Immunologic Specificity of Streptococci Isolated from Various Diseases of Man. EDWARD C. ROSENOW, Division of Experimental Bacteriology, Mayo Foundation, Rochester, Minn.

The following are the chief points which are considered: Methods of preservation of viability and specificity of cultures and bacterial suspensions; methods used in preparation of immune serum; the agglutinating and precipitin reactions of the different strains on isolation after animal passage and after long cultivation on artificial mediums; comparison of immunologic specificity with specific or elective localization in animals.

9. Microbic Dissociation in Streptococci. L. O. DUTTON, Methodist Hospital, Memphis, Tenn.

A description of the various types of dissociating *Streptococcus* colonies. The S type is established and a variety of intermediate and rough forms are considered in an endeavor to identify the true intermediates and the extreme R types. It is pointed out that dissociation in *Streptococcus* strains is marked, and that most of the colony forms described for other bacterial species also occur in *Streptococcus* colonies. Some of the morphological cell changes accompanying the various colony changes are pointed out. The correlation between virulence and the S type, and avirulence and the R types is noted to hold true in natural spontaneous infections.

10. The Interconvertibility of R and S Bacterial Types of B. paratyphosus B by Homologous Immune Sera. M. H. Soule, Hygienic Laboratory, University of Michigan, Ann Arbor, Michigan.

Immune sera were prepared with pure strains of the "S" and "R" forms of *B. paratyphosus B* (209S, 209R, 210S, 210R, Jordan strains). Five-cubic centimeter quantities of plain infusion broth containing various concentrations of immune sera were distributed in test tubes. Serial transfers of each strain were made at forty-eight-hour intervals in homologous and non-homologous serum broth. Before making the transplants a loop of material was removed from each culture and spread on an agar plate. These plates were incubated eighteen hours and examined for the presence of the typical "S" and "R" colonies. "R" forms appeared in strain 209S after 5 serial transplants in homologous serum broth and in the 210S strain after 2 such transfers. "S" types appeared in strain 209R after the seventh transfer in homologous serum and in strain 210R after the sixth transfer. The number of variants in each strain increased with each successive transfer but the cultures never became completely dissociated under these conditions; some residual "S" or "R" organisms always remained. The "R" and "S" forms produced in this manner had all of the characteristics of the original "R" and "S" cultures. No dissociation occurred in the "S" types when grown in the presence of "R" sera (30 transfers). "S" forms appeared in the "R" strains grown in the presence of the non-homologous sera after 16 transfers but the number never became large with continued serial transplanting. The most favorable concentration of sera for inciting dissociation was 10 per cent. No dissociation of the strains was observed when serially cultured in broth containing like quantities of normal serum or in plain infusion broth of pH 6.5, 7.4 and 7.8. Variants appeared, after two weeks' incubation, when large volumes (500 cc.) of broth containing 10 per cent homologous immune sera was inoculated. No dissociation occurred during two months' incubation of like volumes of ordinary broth pH 7.4. Intraperitoneal injections of the original "S" cultures in large doses (500 million) were virulent for guinea pigs. The "R" forms were non-virulent. "S" types obtained by dissociation of the "R" types were as virulent as the original "S" cultures. Guinea pigs immunized by the "R" form were resistant to extremely large doses of the "S" form. The "S" form was more strongly antigenic. The view previously advanced that immune serum has a marked dissociating power on its homologous

germ and a stabilizing action on the heterologous culture type is again demonstrated in this work. Similar studies with other organisms are in progress.

11. Microbic Dissociation of the Tubercle Bacillus. S. A. PETROFF, Research and Clinical Laboratory, Trudeau Sanatorium, Trudeau, N. Y.

Four strains of tubercle bacilli have been studied, viz., Human (H 37), Bovine (B l), Avian (A l) and Bacillus Calmette-Guerin (B. C. G.). All of these organisms have been dissociated and 2 colonies isolated, each having distinct cultural characteristics. The last organism has been more completely studied. From this organism (B. C. G.) R and S colonies have been dissociated and the virulence, cultural characteristics, immunological and antigenic properties have been studied. The R colony appeared to be non-virulent, producing localized disease in guinea pigs which eventually healed, while the S colony always led to generalized tuberculosis.

From the studies of dissociation of these various organisms, it appears that the tubercle bacillus falls in with the rest of the organisms as far as dissociation is concerned.

12. Rate of Bacterial Adaptation. VICTOR BURKE AND CATHERINE ULRICH, State College of Washington, Pullman, Washington.

The modification of bacteria has attained considerable importance of late. The rate of modification has received little attention. Experiments were made to determine how quickly an organism becomes adapted to a new environment.

Staphylococcus aureus was exposed to gentian violet for different periods of time. Then the increased ability of these organisms to grow in the presence of the dye was determined. The experiments demonstrated that *Staphylococcus aureus* shows increased tolerance for the dye after eight hours exposure. It is considered that the organism can probably adapt itself in a shorter period of time than was demonstrated. The change in the organism is considered purely as an adaptation to a new environment and is in no way associated with changes in the life cycle, dissociation of strains, selection of variants or with mutations.

13. Recent Cases of Undulant Fever in New York State. RUTH GILBERT AND MARION B. COLEMAN, Division of Laboratories and Research, New York State Department of Health, Albany, New York.

In a series of fifteen specimens submitted to be examined for evidence

of enteric disease, but with clinical histories more suggestive of undulant fever, the blood serum agglutinated cultures of the melitensis-abortus group. During the course of these investigations, two laboratory infections occurred, one with *B. melitensis*, resulting in a prolonged and severe illness, and the other with *B. abortus*, followed by very mild symptoms. This strain of *B. abortus* was far more virulent for guinea pigs than the strain of *B. melitensis*.

14. Thermal Death Point Studies of Brucella abortus. C. M. Carpenter and Ruth Boak, Diagnostic Laboratory, New York State Veterinary College, Cornell University, Ithaca, N. Y.

Human, porcine and bovine cultures of *Brucella abortus* grown in milk and Hiss serum water were exposed to temperatures of 140°, 142° and 160°F. for various intervals. The suspensions were tested for viability by making cultures and by injecting guinea pigs. It was observed that guinea pigs were more reliable than cultures for determining whether all of the organisms had been killed. In a few instances cultures were negative but guinea pigs became infected. The thermal death point of cultures most virulent for guinea pigs was higher than that of cultures which produced only mild lesions of abortion disease in these experimental animals. No cultures were viable after exposure for twenty minutes to a temperature of 140°F.

15. A Study of Iso-Hemolysins. C. M. Downs, Kenneth Koerber and H. P. Jones, University of Kansas, Lawrence, Kansas.

The statement has been made that iso-hemolysins differ from immune hetero-hemolysins in that they can not be reactivated by the addition of fresh serum. The following study deals with the nature of iso-hemolysins which occur in Type I, II and III sera. Only 45 per cent of the individuals in this series showed hemolysins for any type of cell. Hemolysins in Type I sera were more frequently found for Type III and IV cells than for Type II cells. Hemolysins were rarely demonstrable in serum diluted more than 1 to 1. In those sera having no apparent hemolysins by the usual test, the addition of fresh active but non-hemolytic human or guinea pig complement did not give any hemolysis. The absorption of these sera in the cold and the addition of complement to the treated cells did not give any hemolysis. The hemolysins are apparently absent rather than masked. When cells and complement are added to serum heated to 56°C. a slight amount of hemolysis only

can be demonstrated. However, when the appropriate cells are used for absorption with the heated serum, washed and complement added, a fair degree of hemolysis can be obtained. The hemolysis is not as marked as in the original unheated serum. This seems to point to the occurrence of masked hemolysins in the different types of sera. Cells used for absorption in the cold are actively hemolyzed by the addition of complement. Cells absorbed from either heated serum or from unheated serum in the cold gave no hemolysis unless complement was added.

16. A Study of the Blood Groups among Polynesians. CLARA NIGG, Queen's Hospital, Honolulu, Hawaii, and the University of Kansas, Lawrence, Kansas.

In a series of 413 full-blooded Hawaiians the following percentages of blood groups were found: Group I—36.5 per cent, Group II—60.8 per cent, Group III—2.2 per cent, and Group IV—0.5 per cent. This gives them a race index of 22.7.

Of this group 237 were examined for the agglutinogen described by Simson in his study of the third agglutinating system in human blood and which was found associated with the A agglutinogen (Groups II and IV). Among Hawaiians the incidence of this agglutinogen was 100 per cent and was never found in the absence of A (Groups I and III).

In a small group of full-blooded Samoans the following percentage occurrence was found: Group I—38.8 per cent, Group II—28.9 per cent, Group III—26.3 per cent and Group IV—5.2 per cent. This gives a race index of 1.1.

A study of all available published data in regard to the race index shows its inadequacy for determining interracial relationships on that basis alone, and suggests rather that the percentage occurrence of all four groups must be taken into consideration.

17. The Effect of Urea on Living Cells. NOBLE P. SHERWOOD AND FRANCES DUNMIRE, Department of Bacteriology, University of Kansas, Lawrence, Kansas.

A wide variation occurs between species as to the susceptibility of their red blood cells to hemolysis by means of urea. A percentage of urea giving complete hemolysis is constant for given species, but shows marked variation between species. Species differences depends on at

least 2 of many factors: the fragility of the red blood cells and the toxicity of the urea for the cells.

18. Oral, Subcutaneous, and Intravenous Vaccination with Native Fecal Organisms. LaVerne Barnes, State College of Washington, Pullman, Washington.

Experiments were performed with rabbits to determine: (1) whether there are agglutinins present in normal rabbit's serum for native fecal organisms; and, (2) whether an agglutinin titer for such organisms can be induced or raised by injecting vaccine orally, subcutaneously, and intravenously.

Results indicate that commonly there are no normal agglutinins for native fecal organisms, or if present, they are very weak. As a result of inoculating the vaccine, it appears that the intravenous method is most efficient, and the oral method least efficient in stimulating the production of agglutinins. These results have a bearing on Besredka's theory of local immunity.

19. A Comparison of the Effectiveness of Vaccination against Pasteurella avicida by the Oral, Subcutaneous, and Intravenous Methods. LaVerne Barnes, State College of Washington, Pullman, Washington.

Experiments performed on chickens and rabbits were devised to compare the resistances to infection of the animals injected with avicida vaccine by the oral, subcutaneous, and intravenous methods. The relative effectiveness of each method was determined by administering tests doses of the virulent organisms following immunization.

The results indicate that the intravenous method is most effective in producing agglutinins for the organisms, and in bringing about actual resistance to infection, while the oral method is least efficient. These results have a bearing on Besredka's theory of local immunity.

20. Agglutinin Production. C. A. Behrens and C. H. Keipper, Purdue University, Lafayette, Indiana.

The purpose of this work is to show the relationship between dosage, total amount of agglutinogen injected, and the interval of inoculations. All injections were made intravenously.

Experiments were conducted in which (1) the dose was constant while the total amount of the agglutinogen injected and the interval of in-

oculations were varied; (2) dose and total amount were constant and the interval of injections was varied; (3) dose and frequency of inoculations were constant while the total amount was varied; (4) dose, total amount and interval of injections were constant; (5) dose, total amount and interval of injections were varied; (6) total amount was constant, dose and frequency were varied; (7) total amount and frequency were constant, the dose varied; (8) frequency of injections was constant, dose and total amount were varied.

21. The Preservation of Complement in Various Gaseous Environments with Special Reference to Carbon Dioxide. GEORGE VALLEY, Sterling Research Fellow, Yale University, AND JAMES G. MCALPINE, Storrs Agricultural Experiment Station, New Haven, Conn.

Diluted (1:2) guinea pig serum was subjected to the following gaseous environments: (1) Hydrogen, (2) nitrogen atmosphere obtained by removal of oxygen from air by alkaline pyrogallol solution, (3) vacuum, (4) carbon monoxide, (5) carbon dioxide and (6) ordinary atmosphere (open test tube). The temperature of storage was about 10°C.

The deterioration of the complement which was stored in open tube (cotton plugged) proceeded quite rapidly, the serum losing its hemolytic activity in 5 to 6 days.

In vacuum, hydrogen, and nitrogen the hemolytic titer of the complement changed but slightly in 8 days. During the 19 days of storage the original titer of from 0.01 to 0.03 cc. had changed to 0.2 to 0.3 cc.

In carbon monoxide atmosphere the original titer of 0.01 cc. changed to 0.06 to 0.08 cc. in 21 days, while after 29 days the complementary unit of the stored serum was greater than 1.0 cc.

In case of the carbon dioxide-treated serum the titer was maintained practically unchanged for 29 days. In 3 months a change from 0.03 to 0.05 cc. was observed. From one sample which had an original titer of 0.01 cc. a titer of 0.06 cc. was obtained after 129 days in storage. The replacement of air by carbon dioxide yielded the most satisfactory and promising results, aside from ease of employment. Consequently, storing of the complement in a CO_2-atmosphere has been made a routine procedure in the infectious abortion work at the Storrs Agricultural Experiment Station laboratory, and has been found during the past 8 or 9 months to be an effective and feasible means for preserving serum complement.

The method is simple and is generally carried out as follows: A small

test tube containing either the diluted or undiluted complement is placed in a pint fruit jar having a tight-fitting cover. The top is placed slightly to one side and the end of a delivery tube from a carbon dioxide tank is lowered to the bottom of the jar. Carbon dioxide gas is run in slowly under ½ to 1 pound delivery pressure, and usually for not less than 2 to 3 minutes. The tube is withdrawn and the top clamped down. In order to insure complete or almost complete replacement of air, carbon dioxide application may be repeated, the jar resealed and placed in the ice box. By this method complement may be kept for 2 to 5 weeks with little loss in potency.

The procedure described here furnishes a means of preserving and storing left-over diluted complement as well as fresh undiluted complement for reasonable periods of time, and thus eliminates the necessity of collecting complement daily.

22. The Inheritance of Resistance to the Danysz Bacillus in the Rat.

M. R. Irwin (Introduced by C. H. Werkman), Department of Bacteriology, Iowa State University, Ames, Iowa.

A dose of 15×10^7 organisms of the Danysz bacillus injected intraperitoneally into 131 heterogeneous laboratory rats, pen inbred for 8 years, produced 82.4 ±0.21 per cent mortality. Increasing or decreasing the dose produced a direct increase or decrease in the mortality rate.

Rats of the Wistar Institute stock "A" strain, which have been inbred brother-sister for over 50 generations, when injected with the same dose, gave 98.6 ±0.17 per cent mortality for 69 individuals.

Rats of the Wistar Institute "B" strain, also inbred brother-sister for over 50 generations, gave 80.6 ±1.23 per cent mortality for 31 individuals.

Survivors of heterogeneous rats, mated together, produced progeny which showed a decided increase in numbers of resistant individuals, the mortality being 31.9 ±1.06 per cent for 97 individuals. Survivors of this first resistant generation, mated together, produced a second generation which showed a further increase in resistant individuals, the mortality being 17.0 ±1.06 per cent for 47 individuals tested.

Surviving males of this first resistant generation were mated to nontested females of the susceptible Wistar "A" strain, to sisters of those that had succumbed to the injection, and produced progeny that gave 34.9 ±2.13 per cent mortality for 63 individuals. The results obtained in this progeny eliminate the possibility that the increased resistance for the first and second resistant generations was due to maternal trans-

mission of passive immunity and indicates that it is largely genetic in nature.

23. The Influence of Environmental Changes upon Gastric Secretion and Intestinal Bactericidal Function. LLOYD ARNOLD, Department of Bacteriology, University of Illinois, College of Medicine, and the State Department of Public Health, Chicago, Ill.

The endogenous bacterial flora of the small intestine is almost constant for the mammalian animals so far investigated. The hydrogen-ion concentration within the lumen of this tract has been found to be constant. Environmental factors, both nutritional and meterological, as well as parasitism, cause a maladjustment of the physiological functions and is accompanied by a change in the bacterial flora and chemical constituents of the small intestine. This change can only be interpreted as a part of the mechanism of adaptation of the organism to its environment. We are reporting differences in the bacterial flora of the small intestine obtained from a study of gastric and caecal fistulae dogs placed upon different diets and in rooms with different temperature and humidity. In the process of adaptation to these changes in environment, gastric function and the biological bacterial regulatory mechanism of the small intestine have been studied. In a normal organism there is an auto-disinfecting process in the upper half or two-thirds of the small intestine that destroys the bacteria ingested and prevents the large intestinal flora from extending upwards. This self-regulatory mechanism of the bacterial flora is only one of a group of functions of the splanchnic area in the normal animal. Certain changes in diet, as well as climate, can cause a loss of this bacterial killing power of the intestinal tract. This accompanies a general derangement of several physiological functions of the body.

24. The Duodenal Flora in Achlorhydria. LEO RICEN, H. J. SEARS AND LILLIAN M. DOWNING, Department of Bacteriology, University of Oregon Medical School, Portland, Oregon.

The chief stimulus to the study of this subject lies in the growing importance of achlorhydria in the etiology of pernicious anemia and the conception of that disease as a symptom complex resulting from a slowly acting hemolytic toxin of gastro-intestinal origin.

The material for this study consisted of the duodenal contents of 30 patients who presented the condition of persistent achlorhydria. Two suffered from pernicious anemia, 26 had anemia of the chlorotic type

and only 4 had a fairly normal blood picture. The duodenal contents of 14 normals were also studied. The material in each case was collected with the duodenal tube (Jutte's modification) after thorough cleansing of the mouth and rinsing with 1:2000 potassium permanganate, the final position of the tube in the duodenum being determined by fluoroscopic examination.

Bacteriological examination of the material consisted only in plating on blood agar and recording the appearance of the plates, relative numbers of colonies, etc., and then identifying the various colonies.

The most striking observation was the regular appearance of a rich flora in practically all cases of achlorhydria and the almost complete absence of organisms in the specimens from the normals. In the achlorhydric material blood destroying organisms were extremely common and included hemolytic streptococci and staphylococci as well as hemolytic *B. coli* and other Gram negative bacilli. Green producing streptococci and other green producing colonies were observed frequently. *B. alcaligenes* was noted several times, pneumococci twice and Gram negative diplococci occasionally. *B. coli* was prominent in both of the pernicious anemia cases.

Persistent achlorhydria is conducive to a copious growth of aerobic bacteria in the duodenum and this abnormal flora contains forms representative both of the mouth and of the lower levels of the digestive tube.

25. Epidemic Encephalitis in Foxes. R. G. Green, N. R. Zieglar, H. O. Halvorson, B. B. Green and E. T. Dewey, Department of Bacteriology, University of Minnesota, Minneapolis, Minn.

During the past three years an exhaustive study has been made of a disease in epidemic form occurring on silver fox farms. Deaths in a single epidemic have totaled more than 700. The disease is characterized typically by weakness, convulsions and a lethargic state. The onset is usually sudden, the animal dying in from one to three days after the onset. Gross pathology is not marked, consisting mainly of hemorrhages in various organs, including brain and spinal cord. Microscopic pathology of the brain and spinal cord show abnormal collections of cells, more frequently about the blood vessels; hemorrhages into the surrounding tissues are common. The disease has been transmitted consistently by the injection of the brain and spinal cord taken from a sick animal, into well foxes, by cisterna puncture or intracranial injection. Whereas the mortality of the natural disease is from 10 to 25

per cent of animals on a ranch, the mortality in artificially infected animals is close to 100 per cent. The exact nature of the infective agent is as yet undetermined, although a streptococcus is under suspicion. The virus appears to be neutralized by castor oil soap. Animals vaccinated by means of a soap virus mixture appears to have resistance to artificial infection.

(Note: The group carrying out this work wishes to acknowledge the aid, since October 1, 1927, of Dr. J. E. Shillinger, of the Bureau of Biological Survey.)

26. Symptoms of Epidemic Encephalitis of Foxes. R. G. GREEN, Department of Bacteriology, University of Minnesota, Minneapolis, Minn.

A portion of the moving picture records of both ranch animals and experimental animals used in the study of encephalitis of foxes.

27. Hemorrhagic Septicemia of Fish. DAVID L. BELDING, Boston University School of Medicine, Boston. Mass.

Fish septicemia, a disease of considerable economic importance to fish culturists in the New England and Middle Atlantic States, is reported in several Massachusetts hatcheries. The causative organism is a member of the *Pasteurella* group apparently identical with *B. salmonicida*, first described in Bavaria by Emmerich and Weibel. Several strains have been isolated from hatchery and wild fish. The disease seems specific for fish, since attempts to inoculate other poikilothermal animals have proved unsuccessful. No species of salt or fresh water fish common to New England waters is immune. Infection is transmitted by contact, contaminated water, and ingestion.

The type of disease is determined by the rapidity of death. The epidemic form is characterized by a septicemia without local lesions, and non-epidemic form by necrotic areas in the muscles and subcutaneous tissue. No satisfactory prophylactic treatment has been discovered. Certain epidemics have been held in control by keeping the water below 55°F.

28. A Pleomorphic Anaerobe: The Etiological Organism in a Fatal Meningitis. EDNA G. JACKSON, Henry Ford Hospital, Detroit, Mich.

A markedly pleomorphic, anaerobic, Gram-negative bacillus is described which was isolated from a fatal case of meningitis. The

organism was obtained from cultures of the spinal fluid, also from blood cultures taken before death and at autopsy. It is virulent for laboratory animals and this virulence has been retained after several months' growth upon artificial media. The cultural characteristics are described, also the results of animal inoculation.

29. Colloid Chemical Aspects of the Kahn Reaction. R. L. KAHN, Michigan Department of Health, Lansing, Michigan.

The correlation of the author's observations on the precipitation phenomenon with syphilitic serum especially with reference to the Kahn reaction, with factors which govern other colloidal precipitation reactions.

30. Principles of Antigen Correction for Kahn Reaction. R. L. KAHN, ELIZABETH MCDERMOTT AND GRACE LUBIN, Michigan Department of Health, Lansing, Michigan.

Over 4 years ago, an antigen having a certain degree of sensitiveness with syphilitic serum was selected on the basis of clinical and serological studies, as standard for the routine Kahn test. All antigens prepared since then have been made to conform to this standard. Antigens prepared from different lots of heart muscle according to the prescribed method, may have a degree of sensitiveness greater or less than the standard. The sensitiveness of a given antigen at its titer is closely related to its lipoid content. Both dilution and concentration of an antigen when continued to excess, will ultimately decrease its sensitiveness with serum, maximum sensitiveness for any antigen being associated with optimum lipoid content. Thus, if the lipoid concentration of an antigen at its maximum sensitiveness is increased or decreased 25, 50, 75 and 100 per cent it will be found that the greater the change in concentration in either direction, the greater the reduction in sensitiveness. These facts form the basis for antigen correction.

If an antigen is less sensitive than standard requirements, presumably it is either too rich or too poor in lipoids. If too rich, it is diluted with cholesterolized alcohol; if too poor—and this can be readily recognized from its behavior with salt solution—it is concentrated by the addition of lipoids. The increase or decrease in concentration required to bring such an antigen to standard sensitiveness can be determined by making Kahn tests with syphilitic and non-syphilitic serum, using standard antigen as a control. If a new antigen is more sensitive than standard requirements, its sensitiveness may be reduced by increasing or by

decreasing its concentration. Since reducing the concentration of the antigen by diluting with alcohol is technically simpler than concentrating it, the former method is usually resorted to. After correction an antigen does not require re-titration but may be used in a titer of 1 cc. antigen + 1.1 cc. saline.

It should be emphasized that an antigen may be reduced to dryness by evaporation and the residue taken up in a volume of alcohol corresponding to that of the original antigen, without decreasing the sensitiveness.

A NOTE ON A LIGHT-ACTIVATED HEMOLYSIN PRODUCED BY B. INFLUENZAE

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While searching for rough and smooth colony variations of *B. influenzae* on various solid media, a curious phenomenon was observed when the organism was grown on fresh blood agar plates enriched by adding the "X" and "V" factors of Thjötta and Avery (1921). According to Fildes (1923), the "X" factor can be supplied by either hematin or potato, and the "V" factor by animal or vegetable cells—blood cells or yeast being ordinarily used. As is well known, *B. influenzae* will grow much more luxuriantly on heated (chocolate) blood agar than on fresh blood agar, the heated blood supplying both hematin and the "V" factor. In the course of the study of colony variations of *B. influenzae* it was desirable to employ a transparent medium. For this reason, pepsin-digested blood (Fildes, 1920) was used instead of the heated blood, and although this gives fairly large colonies, growth was further enhanced by the addition of yeast extract. *B. influenzae* grows very poorly on fresh horse blood agar plates, but if pepsin-digested blood and yeast extract are added, the growth is excellent. Such plates are very similar in appearance to ordinary fresh blood agar plates and any degree of hemolysis, if present, can be clearly and easily seen.

We were studying media of this kind and their modifications, chiefly for purposes of colony observations, when we encountered the phenomenon which forms the subject of this paper.

With the exception of a few hemolytic strains, colonies of *B. influenzae*, when grown on fresh blood agar plates, do not show any area of hemolysis surrounding the colonies. If the organism

is sown on fresh horse blood agar plates enriched by pepsin-digested blood and yeast extract, not a trace of hemolysis is visible when the plates are taken out of the incubator. If these plates are then exposed to light, however, a well-marked area of clear hemolysis appears surrounding each colony, after a length of time which depends on the intensity of the light. If exposed to sunlight, the hemolysis begins to appear after about an hour and a quarter, but on a dull day no hemolysis will be seen for some hours. Control plates, either kept in the incubator or at room temperature in the dark, show no change. Ultra-violet light from a Mercury vapor lamp appeared to be less active than sunlight filtered through two thicknesses of ordinary glass. Many strains of *B. influenzae* have been tested and all have shown the same phenomenon, although it is more marked with some strains than others. In the course of the main study on rough and smooth colony variations of *B. influenzae* many rough forms were found and were tested. A great many other organisms were grown on the same medium, but the phenomenon was seen only in the case of *B. influenzae* and appears to be characteristic. One throat culture on this medium showed a single colony of *B. influenzae* which was identified by this method.

By the process of exclusion, it was found that the hemolysis did not occur in the absence of the pepsin-digested blood. If the pepsin-digested blood was replaced by chocolated blood, hemolysis occurred after exposure to light, but was not so clear-cut owing to the greater opacity of the medium. As hemoglobin is changed into hematin both by heating and digestion with pepsin, it appeared probable that pure hematin could be used in place of the pepsin-digested or heated whole blood. Accordingly, yeast extract and a solution of pure hematin were added to fresh horse blood agar, and *B. influenzae* when grown on this medium showed the characteristic hemolysis after exposure to light.

Having found that hematin was the essential ingredient of the medium, it was naturally of interest to determine whether the light sensitized the red cell to hemolysis, or whether the light changed some substance excreted by the organism into a true hemolysin. The latter hypothesis seems the more likely explana-

tion, especially as the red cells are not made more sensitive by long exposure of the plates to light before incubation, but it may well be that the change is a complex one. As will be seen later, the hematin alone produces a slow change tending towards hemolysis of the red cells. If the phenomenon could be produced with broth cultures or with filtrates of these acting on red cells, the mechanism of the reaction could be studied more easily, but all attempts to do this have failed.

The medium which has proved the most satisfactory is made as follows:

Two per cent hormone agar is melted, cooled to 45°C. and 5 per cent of fresh defibrinated horse blood, 10 per cent of yeast extract and 5 per cent of pepsin-digested blood added. The plates are then poured. The pepsin-digested blood should be added just before pouring the plates, and care taken that the temperature is not above 45°C. If the temperature is too high when the ingredients are mixed, the plates are apt to undergo spontaneous hemolysis. When poured, the plates should be kept in the ice-box if not used at once. Kept in this way, they will not deteriorate for some days; but after a week in the ice-box, they may hemolyze spontaneously on incubation. This change is doubtless caused by the hematin. The plates will still undergo this slow change towards hemolysis if the pepsin-digested blood is boiled before adding to the medium, thus removing any suspicion that an excess of pepsin is responsible.

The pepsin-digested blood is made in the manner described by Fildes (1920).

Into a ground-glass-stoppered, sterile bottle one places in the following order 250 cc. of saline, 6 cc. of pure hydrochloric acid, 50 cc. of defibrinated horse blood and 1 gram of pepsin. The bottle is shaken to dissolve the pepsin, and incubated in a 55°C. water-bath for about twenty hours, shaking occasionally at first. At the end of this time add 12 cc. of 20 per cent NaOH, and check the reaction with phenol red and cresol red. As the fluid is a very dark brown, it has to be well diluted with distilled water when titrating. A pH of about 7.2 should be aimed at, i.e., a distinct red with phenol red and no change with cresol red. Sterile precautions should be taken when removing the fluid for titration. It is best to make sure of a red color with cresol red by adding more NaOH

if 12 cc. is not sufficient, and then going back with hydrochloric acid. When the proper pH has been reached, add 0.25 per cent of chloroform and grease the stopper with sterile vaseline before replacing. Shake the bottle well and store in the cold. It will keep for some months, if the pH is correct, but if too alkaline it will deteriorate more rapidly, and if too acid it will precipitate the haematin. There is no necessity to remove the chloroform before adding to the medium.

In place of the pepsin-digested blood, chocolate blood may be used and the plates made by adding 1 cc. of defibrinated horse blood to the agar, heating to 80°C. for a few minutes, cooling the mixture to 45°C., adding 5 per cent fresh defibrinated horse blood, and pouring the plates. The results are not so clear-cut as with the pepsin-digested blood plates.

The yeast extract is made in the manner described by Avery and Thjötta (1921).

One hundred grams of yeast are emulsified in 200 cc. of water, and after acidifying to a pH of about 4.6, boiled for ten minutes. The emulsion is either allowed to stand for twenty-four hours or centrifuged at once, the supernatant fluid taken off under sterile conditions and placed in a sterile glass-stoppered bottle. The reaction is adjusted to a pH of 7.0 and 0.25 per cent of chloroform is added. Grease the stopper with sterile vaseline and shake the bottle. If kept in the cold, this extract will also remain active for some months. The chloroform need not be removed before adding the extract to the medium.

CONCLUSIONS

B. influenzae, if grown on fresh horse blood agar plates containing hematin, shows no hemolysis surrounding the colonies after incubation in the dark, but if the plates are then exposed to sunlight or daylight or artificial light, an area of clear hemolysis appears around the colony after a length of time depending on the intensity of the light.

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THE ACTION OF B. DIPHTHERIAE AND SOME RELATED ORGANISMS ON GLUCOSAMINE

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Glucose and the amino group both play important rôles in bacterial metabolism, yet the ability of different bacteria to utilize glucosamine has been given little study.

Abderhalden and Fodor (1913) reported that *B. tenuis* of the subtilis group, contained in a piece of putrid pancreas, formed propionic and lactic acids from glucosamine. They suggested that, following deamination, glucose was formed, and subsequently fermented. Their culture medium consisted of 3 per cent glucosamine hydrochloride, 0.25 per cent Witte's peptone, 0.5 per cent glucose, and magnesium sulphate, the whole buffered with sodium phosphate. Their reason for incorporating glucose is not apparent; in fact its presence might have protected the glucosamine from bacterial action; certainly one could not readily determine whether changes occurring in the medium were due to splitting of the glucosamine, or of the glucose. Meyer (1913) used a medium of glucosamine hydrochloride with casein solution, in fermentation tubes. He reported that the organisms with which he worked, fermented glucosamine in a way analogous to their action on glucose; that is, with acid alone, or with both acid and gas. Meyer (1914) in later work, subjected Abderhalden and Fodor's hypothesis of preliminary deamination to experimental test. He used monacetylglucosamine ($C_6H_{11}O_5-NH-CO-CH_3$) instead of glucosamine hydrochloride, as in his earlier work, and found that the substitution of the monacetyl radical for the amino group in the glucosamine molecule, resulted in the failure of many organisms to ferment. He concluded that the primary point of attack

[1] Some of the work herein reported was submitted by the junior author in partial fulfillment of the requirements for the degree of Master of Science.

on glucosamine was not at the bond connecting the amino group, and since *B. paratyphosus* A produced acid but no gas, from glucosamine hydrochloride, that glucose could not be formed as an intermediate product.

We have found no reference to study of the action of *B. diphtheriae*, and its related bacteria, on glucosamine. The differentiation of the virulent from the non-virulent members of the group is often a public health problem of considerable importance. We have, therefore, undertaken a study to determine whether any of these organisms can utilize glucosamine, and whether differences exist in their action which might be correlated with their ability to form toxin.

Glucosamine is an unstable base, but it forms stable salts quite readily, the hydrochloride being easily obtained in the acid hydrolysis of lobster shells, and other chitinous substances. We secured samples of the hydrochloride from different manufacturing chemists, and made tests to determine their approximate composition and purity.

None of the samples was fermented by yeast, thus indicating that glucose was not present as an impurity. These results agree with those reported by Thorp (1921), Hammarsten (1914), and Ledderhose (1878; 1880). On heating with phenyl hydrazine, typical glucosazone crystals were formed (Thorp, 1921; Hammarsten, 1914; Speilman 1892). The total nitrogen as determined by Kjeldahl's method agreed closely with the theoretical nitrogen of the molecular formula. It therefore seemed that the substance with which we were working was reasonably pure glucosamine hydrochloride.

TECHNIQUE

For the preliminary cultural tests, the following basic media were prepared: (*a*) 2 per cent solution of Park Davis peptone water, (*b*) diphtheria toxin broth, (*c*) Hiss serum water. The peptone water was made in the usual way, and the reaction adjusted to pH 7.6. The diphtheria toxin broth was prepared according to Smith's method, and the reaction also adjusted to pH 7.6. The Hiss serum water was made from horse serum,

TABLE 1

ORGANISM	FERMENTATION			VIRULENCE IN GUINEA PIGS
	Glucose	Sucrose	Dextrin	
1. *B. diphtheriae*, Park-Williams No. 8	+	−	+	Virulent
2. *B. diphtheriae* A, recently isolated from a clinical case of diphtheria	+	−	+	Virulent
3. *B. diphtheriae* No. 10, recently isolated from a clinical case of diphtheria	+	−	+	Virulent
4. *B. diphtheriae*-like B, recently isolated from a suspected carrier	+	−	+	Non-virulent
5. *B. hofmanni*, old culture	−	−	−	Non-virulent
6. *B. hofmanni*, recently isolated	−	−	−	Non-virulent
7. *B. xerosis*	+	+	−	Non-virulent
8. *B. diphtheriae*-like H_1 (Typical morphology)	+	−	+	No protection by diphtheria antitoxin. Ulcer formed at point of inoculation of both toxin and antitoxin pigs
9. *B. diphtheriae*-like W_1 (Typical morphology)	+	−	+	No protection by diphtheria antitoxin. Ulcer formed at point of inoculation of both toxin and antitoxin pigs
10. *B. diphtheriae*-like H_2 (Typical morphology)	+	−	+	No protection by diphtheria antitoxin. Ulcer formed at point of inoculation of both toxin and antitoxin pigs
11. *B. diphtheriae*-like W_2 (Typical morphology)	+	−	+	No protection by diphtheria antitoxin. Ulcer formed at point of inoculation of both toxin and antitoxin pigs
12. *B. diphtheriae*-like Ha (Typical morphology)	+	−	+	No protection by diphtheria antitoxin. Ulcer formed at point of inoculation of both toxin and antitoxin pigs

+ = acid production; − = no acid production.

which was rendered sugar free by fermentation with *B. coli*. The reaction of this last medium was found to be about pH 8.0, and was left unchanged. These media were sterilized by steam heat, in bulk, and an equal volume of 2 per cent solution of glucosamine hydrochloride, adjusted to pH 7.6 was then added. This glucosamine hydrochloride solution had been previously sterilized by passing it through a Mandler filter.

Tests were made to determine a suitable indicator, since Andrade's acid fuchsin indicator did not give sufficiently definite color changes to be of practical use for our work. Brom thymol blue (0.1 per cent of a 1.6 per cent solution in ethyl alcohol) was tried, but it was found to have too long a scale of intermediate color changes to be satisfactory. Brom cresol purple (0.1 per cent of a 1.6 per cent solution in ethyl alcohol) was finally adopted, since it showed the most definite color changes. Moreover, its color changes occurred at lower points on the pH scale (pH 5.2 to pH 6.8) and since the initial reaction of our media was pH 7.6 (except the Hiss serum water, which was pH 8.0) color changes could only occur when definite fermentation of the glucosamine took place.

The media, with the indicator added, were finally pipetted with sterile technique into 3-inch-by-$\frac{3}{8}$-inch test tubes, 1 cc. in each tube. All the glassware had first been carefully neutralized.

Parallel tests were made to determine whether (1) glucosamine hydrochloride in 1 per cent solution, adjusted to pH 7.5 to 7.6, and (2) the three basic media (peptone water, toxin broth, and serum water) would support satisfactory growth of the organisms selected for study, and (3) to compare changes in the basic media containing glucosamine with changes in the same media containing glucose.

In early tests two cultures were employed, *B. diphtheriae* (Park-Williams No. 8) and *B. hofmanni*. Later it was found desirable to include additional strains of the same group. The seeding cultures were grown for twenty-four hours on Loeffler's coagulated serum medium, and one loopful was then inoculated into the different media. The tests were made in triplicate and incubated for forty-eight hours at 37°C. Readings were then

TABLE 2

ORGANISMS	TESTS						CONTROLS											
	Peptone water + glucosamine 1 per cent			Serum water + glucosamine 1 per cent			Peptone water			Peptone water + glucose 1 per cent			Hiss serum water			Hiss serum water + glucose 1 per cent		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
B. diphtheriae Park-Williams No. 8	+	+	+	+c	+c	+c	−	−	−	+	+	+	−	−	−	+c	+c	+c
B. diphtheriae A	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+c	+c	+c
B. diphtheriae No. 10	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+c	+c	+c
Bd-like B	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+c	+c	+c
B. hofmanni	−	−	−	*			−	−	−	−	−	−	*			−	−	−
B. hofmanni C	−	−	−	*			−	−	−	−	−	−	*			−	−	−
B. xerosis	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+,±c	+,±c	+,±c
Bd.-like H_1	+l	+	+l	−	−	−	−	−	−	+	+	+	−	−	−	+c	+c	+c
Bd.-like W_1	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+c	+c	+c
Bd.-like H_2	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	+,±c	+,±c	+,±c
Bd.-like W_2	−	+	−	−	−	−	−	−	−	+	+	+	−	−	−	+c	+c	+c
Bd.-like H_3	+	±	+	−	−	−	−	−	−	+	+	+	−	−	−	+,±c	+,±c	+,±c

+, +l, ± indicate intensity of acid production; + = complete change of color—purple to yellow; +l = almost complete change of color—purple to grayish-yellow; ± = slight change of color—purple to gray with purple tinge.

− = no acid production—color unchanged; c = coagulation; ± = slight coagulation; 1, 2, 3 = number of test; Bd = *B. diphtheriae*.

* No growth.

Note: Only one tube of a triplicate test appearing to show no growth was subcultured. The words "No growth," therefore appear only once. This rule was followed in subsequent experiments unless otherwise indicated.

made for color changes, and for coagulation in the serum medium. Wherever growth appeared questionable, transplants were made onto Loeffler's medium.

These preliminary tests showed that the unbuffered glucosamine solution did not support growth; the peptone water, toxin broth, and Hiss serum water gave satisfactory growth, although the peptone water cultures were at times rather light. In peptone water, toxin broth, and Hiss serum water, all with glucosamine added, acid color changes were noted in cultures of *B. diphtheriae* (Park-Williams No. 8), but these changes were more pronounced when the media contained glucose, instead of glucosamine. Evidently therefore, *B. diphtheriae* could split glucosamine, provided a medium was employed which was able to support a sufficiently heavy growth. It will be seen in later tests that not all strains of *B. diphtheriae* can do this.

These results encouraged us to make further tests, with additional cultures. Peptone water, and Hiss serum water, both with glucosamine added, were selected as the media. The organisms used were three virulent cultures of *B. diphtheriae*, Park-Williams strain No. 8, and strains "A" and No. 10, the last two recently isolated from clinical cases of diphtheria; strain "B," fermenting glucose and dextrin, but non-virulent; two strains of *B. hofmanni; B. xerosis;* and five strains of organisms resembling *B. diphtheriae*, but producing local non-specific, superficial skin necrosis on injection into guinea pigs, the necrosis not being prevented by diphtheria antitoxin.

The fermentation and pathogenic characteristics of these organisms are listed in table 1.

The manner in which these organisms act on the different culture media is shown in table 2.

It will be noted from this table that different strains of *B. diphtheriae* varied in their ability to act on glucosamine, the Park-Williams strain No. 8 alone being able to ferment it. The six strains of *B. diphtheriae*-like organisms, also varied; strains H_1, W_2, and H_a, produced acid irregularly, while strains B, H_2, and W_2, brought about no change in glucosamine. Both strains of *B. hofmanni* failed to grow in the Hiss serum water unless glucose was added.

TABLE 3

ORGANISMS	TESTS												CONTROLS			
	Serum-water + 1 per cent peptone + 1 per cent glucosamine						Toxin broth + glucosamine 1 per cent						Serum water + 1 per cent peptone	Serum water + 1 per cent peptone + 1 per cent glucose	Toxin broth	Toxin broth + 1 per cent glucose
	1	2	3	4	5	6	1	2	3	4	5	6				
B. diphtheriae Park-Williams No. 8	+c	+c	+c	+c	+c	+c	+	+	+	+l	+	+	−	+c	−	+
B. diphtheriae A	−	−	−	−	−	−	−	−	−	−	−	−	−	+c	−	+
B. diphtheriae No. 10	Tr.	Tr.	Tr.	−	−	−	−	−	−	−	−	−	−	+c	−	+
Bd.-like B	−	−	−	−	−	−	−	−	−	−	−	−	−	+c	−	+
Bd-like No. 11	−	−	−				−	−	−				−	+c	−	+
B. hofmanni	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. hofmanni C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B. xerosis	−	−	−	−	−	−	−	−	−	−	−	−	−	+c	−	+
Bd.-like H_1	+l, ±c	+l, ±c	+l, ±c	±	±	±	−	−	−	−	±	±	−	+c	−	+
Bd.-like W_1	±	±	±	−	−	−	−	−	−	−	−	−	−	+c	−	+

+, +l, ± indicate intensity of acid production; + = complete change of color—purple to yellow; +l = almost complete change of color—purple to grayish-yellow; ± = slight change of color—purple to gray with purple tinge.

− = no acid production—color unchanged; c = coagulation; ± = slight coagulation; 1, 2, 3 = number of test; Bd = *B. diphtheriae*.

Tr. = trace of acid indicated by a very slight fading of color.

In view of these results, we decided to try toxin broth, and Hiss serum water modified by adding to it 1 per cent peptone, in the hope that better growth might thus be obtained, and conditions rendered more favorable for fermentation of the glucosamine. Another *B. diphtheriae*-like organism, strain No. 11,

TABLE 4

ORGANISMS	TESTS: Casein medium + glucosamine 1 per cent							CONTROLS: Casein medium		Casein medium glucose 1 per cent	
	1	2	3	4	5	6	Fermentation tube 7	3 inch x ¼ inch	Fermentation tube	3 inch x ¼ inch	Fermentation tube
B. diphtheriae Park-Williams No. 8....	−	−	−	−	−	−	− P3	−	−	−	−
B. diphtheriae A.....	−	−	−	−	−	−	− P2	−	−	−	−
B. diphtheriae No. 10	−	−	−	−	−	−	− P3	−	−	Tr.	−
Bd.-like B.....	−	−	−	−	−	−	− P2	−	−	−	Tr. bulb, − arm
Bd.-like No. 11.....	−	−	−	−	−	−	− P2	−	−	−	−
B. hofmanni.....	−	−	−	−	−	−	− P2	−	−	−	−
B. hofmanni C.....	−	−	−	−	−	−	− P2	−	−	−	−
B. xerosis.....	−	−	−	−	−	−	− P1	−	−	−	−
Bd.-like H_1.....	±	±	±	Tr.	Tr.	Tr.	± bulb, − arm, P4	−	−	±	+ bulb − arm, P1
Bd.-like W_1.....	Tr.	Tr.	Tr.	−	−	−	± bulb, − arm P4	−	−	+	+ bulb, + arm, P1

P = precipitation of casein; 4, 3, 2, 1 = degree of precipitation.

was added to the strains used in our previous tests. This strain was similar morphologically and culturally to *B. diphtheriae*, but was non virulent, and did not produce skin necrosis on injection into guinea pigs. Strains H_2, W_2, and H_3, were not employed in any of these later tests. The results of two sets of

tests, each in triplicate, are combined in table 3, the individual tests being numbered from 1 to 6. The control tests were done in duplicate, but are recorded only once, in order to save space.

In the tests recorded in table 3, it was observed that the two media used in this series supported more satisfactory growth; the Hiss serum water to which 1 per cent peptone had been added, gave greatly improved growth, as compared with serum water without peptone; and the toxin broth gave a heavier growth than the peptone water used in the earlier tests (table 2). The heavier growth resulted in stronger fermentation of the glucosamine, and also in its fermentation in this series, by *B. diphtheriae* No. 10, a strain which in the previous series (table 2) had been inactive towards it. The Park-Williams strain No. 8 fermented it very definitely and regularly, being the most active in this respect of all of our cultures. Both strains of *B. hofmanni* grew satisfactorily in this series.

Our results thus far with the different media had been less definite than we had hoped. Meyer (1913) however, had reported definite reactions on glucosamine in a casein medium. He had employed various bacteria, including members of the colon-typhoid group, but not *B. diphtheriae* or related organisms. We therefore, decided to try his medium with the group of organisms we were studying. We substituted brom cresol purple as the indicator, in place of litmus, but in all other respects followed his methods exactly. Our results are shown in table 4.

For *B. diphtheriae* and related organisms, Meyer's casein medium gave less satisfactory growth than either toxin broth, or the Hiss serum water with peptone added. The reactions on glucosamine were practically negative. *B. diphtheriae* Park-Williams No. 8 had fermented glucosamine consistently in the media used in earlier tests, but in Meyer's medium it failed to ferment either glucosamine, or glucose, probably because of scanty growth.

DISCUSSION

It would appear that glucosamine is fermented with greater difficulty than is glucose. This is not surprising when we consider

the more complex structure of the former substance, and the added probability that deaminization must take place before fermentation can occur. We tried to determine whether the amino group could be utilized as a primary source of nitrogen, but all our strains failed to grow in a plain solution of glucosamine, and it was necessary to add peptone or serum (as in the Hiss serum water) before growth would take place. Both these substances contain nitrogen in a readily available form, and this is undoubtedly the reason for their ability to support growth. Apparently glucosamine is not a primary source of nitrogen, at least for the organisms we have used.

The intensity and regularity with which the Park-Williams strain No. 8 fermented glucosamine (except in Meyer's casein medium) are the outstanding observations in our work. Although it is an old strain, it has retained its power to produce toxin after many years of artificial cultivation, and is still the standard strain for toxin production in many laboratories. Its ability to ferment glucosamine distinguishes it from the other virulent strains used, all of them more recently isolated. We did not attempt to compare quantitatively its toxin production with that of the other strains of diphtheria bacilli, but the observation that two non-virulent strains—non-specific necrosis producers—fermented glucosamine, although not so definitely as the Park-Williams strain No. 8, appears to indicate that there is no correlation between the ability, of the organisms employed, to produce toxin, and to ferment glucosamine.

SUMMARY

In the work reported, we have studied the fermentative reactions on glucosamine of three strains of *B. diphtheriae*, and ten strains of diphtherioids, including one strain of *B. xerosis*, and two of *B. hofmanni*.

Plain solutions of glucosamine failed to support growth, so it would appear that, at least for the organisms studied, glucosamine cannot be utilized as a primary source of nitrogen.

It was found that the addition of 1 per cent peptone to Hiss serum water promoted heavier growth, and more definite fermentation of both glucose and glucosamine.

Glucosamine was not so easily fermented as glucose.

B. diphtheriae Park-Williams No. 8 was the only strain in the group studied which fermented glucosamine definitely and consistently in all tests. Four of the diphtheroids which produced non-specific skin necrosis in guinea pigs, fermented glucosamine irregularly and with varying intensity. The strains of *B. xerosis*, and *B. hofmanni*, used had no action on glucosamine.

These observations show that there is no correlation between ability to ferment glucosamine and to produce diphtheria toxin.

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RELATIVE IMPORTANCE OF ADDITIVE AND ANTAGONISTIC EFFECTS OF CATIONS UPON BACTERIAL VIABILITY

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OBJECT OF THE INVESTIGATION

Studies conducted by numerous investigators, in widely separated fields of biology, have revealed phenomena which are commonly grouped under the general head of salt antagonism. In examining the effect of electrolytes upon heart muscle and sea-urchin eggs, upon chlorophyll-bearing plants and bacteria and even upon non-living organic materials, effects have been noted which are roughly analogous, although the analogy may sometimes be more apparent than real. The essential phenomenon involved is the neutralization by one cation of the toxic effect produced by another; and the commonest case recorded is the antagonism between monovalent and bivalent ions. Loeb (1912) has fully reviewed the earlier literature of the subject and Falk (1923) has presented a more recent summary, particularly in regard to the effects of salts upon bacteria.

In discussing the antagonistic influence of salts on animal tissues and algae and higher plants, it has generally been assumed that antagonism was related to specific and different properties of the different ions concerned. Thus, Loeb (1918) attributed the swelling of protein caused by dilute NaCl to the formation of an ionizable protein salt and the neutralizing influence of $CaCl_2$ to the formation of a non-ionizable salt. Osterhout (1915, 1917 and 1922) similarly assumed a specific increase of permeability of pl nt cells as due to Na and a decrease as due to Ca.

In the field of bacteriology, the chief studies of salt antagonism

[1] Based on thesis submitted by the junior author in partial fulfilment of requirements for the degree of Doctor of Philosophy in Yale University.

have been those of Flexner (1907) on the viability of the meningococcus, of Li ɔman (1909, 1910a and 1910b) on ammonification by *B. subtilis*, of Eisler (1909) on the viability of various species of bacteria, of Loew (1910), Kelley (1912) and Lipman (1911 and 1912) on ammonification, of Lipman and Burgess (1914a and 1914b) on nitrification and nitrogen fixation, of Brown and Hitchcock (1917) on nitrification and Brown and Johnson (1918) on ammonification, of Fulmer (1918) on the growth of bacteria in soil, of Shearer (1917 and 1919) on the viability of the meningococcus and the electrical resistance of a suspension of this organism, of Winslow and Falk (1918) on the viability of *Bact. coli* in water, of Brooks (1920a, 1920b, 1921) on CO_2 production by *B. subtilis*, of Holland (1922), and of Winslow and Falk (1923) on viability of *Bact. coli* and of Shaughnessy and Winslow (1927) on the diffusion products of *Bact. coli*. The evidence for antagonism is on the whole much less clear in these experiments than in those conducted with algae or animal tissues. In general, however, it appears from them that dilute Na tends to favor viability and biochemical activity while a similar concentration of Ca exerts an opposite effect. Mixtures of a monovalent and a bivalent salt or of two univalent salts were found to be more favorable than either one alone by Lipman (1909) Eisler (1909) Lipman and Burgess (1914a) Shearer (1917 and 1919) Winslow and Falk (1918) Brooks (1920a, 1920 and 1921) and Winslow and Falk (1923). The antagonistic effect was manifest in solutions containing five to twenty times as much of the monovalent as of the bivalent cation.

Meanwhile, a different type of studies was conducted by another group of investigators which may perhaps throw considerable light on the problems involved. These were investigations dealing, not with antagonism directly, but with the effect of single cations in varying concentration upon bacterial life and development. The work of Eisenberg (1918), Holland (1922) and Hotchkiss (1923) along this line has brought out the somewhat surprising fact that, in their direct effect upon viability, the various cations ppear to differ in degree rather than in kind. Even such toxic salts as Pb and Hg are stimulating rather than

inhibitory when present in sufficiently high dilution; and even Na, the most favorable of all cations to bacterial life, is toxic in sufficiently high concentration. More recently, Shaughnessy and Winslow (1927) have shown that weak solutions of either Na or Ca (much more dilute of course in the latter case) apparently increase permeability while stronger solutions decrease it.

A consideration of these two lines of evidence led us to suspect that,—so far as their effect upon viability and permeability is concerned,—the influence of cations upon the bacterial cell, alone or in combination, might perhaps be explained without attributing specific and characteristic properties to any particular cation, but merely on the assumption that the cations differ quantitatively in their activity.

In considering this problem it is important to note that the term "salt antagonism" has been used by different observers in very different senses and demonstrated by different kinds of evidence. First of all, salts have sometimes been said to be antagonistic merely because in a similar concentration the pure salts show opposite effects and when mixed neutralize each other. Thus, for example, a 0.1 M solution of NaCl or KCl is generally stimulating, a similar solution of $CaCl_2$ or $MgCl_2$ generally toxic. A mixture of a monovalent and a bivalent salt in this concentration will of course give an intermediate effect. This phenomenon is simply an expression of the quantitative difference in the activity of the two salts and is not properly speaking antagonism at all, any more than the mixture of a toxic concentration of NaCl with a stimulating concentration of the same salt would be antagonism.

Secondly, some investigators have conducted studies on antagonism by using a solution of constant total molality but with varying proportions of two component salts. Such an experiment will generally show that some combination of the two salts is more favorable than either one alone. As our own data will indicate, however, such a result is sometimes explicable as due solely to the direct influence of the dissociated cations present, with no assumption other than that of the demonstrated quantitative difference in activity between the monovalent and bivalent ions.

Thirdly, we have to consider what is really the crucial case, that in which the mixture of two salt solutions, each by itself definitely toxic, produces a non-toxic mixture. Osterhout has defined antagonism in this sense as occurring "when one toxic substance acts as an antidote to another." This is the true antagonism which appears to occur when salts act on the cells of animals and higher plants and which is probably manifest when salts act on certain biochemical reactions set up by bacteria. So far as influence on the viability of bacteria is concerned, however, the evidence for such true antagonism is somewhat scanty as will be pointed out in a succeeding paragraph. Furthermore, before real antagonism can be demonstrated we must take into account (*a*) reactions between the salts and any constituents of the medium which may tend to remove active cations and (*b*) changes in the hydrogen ion content of the menstruum, since Winslow and Lochridge (1906) and many later workers have shown that with acids and simple mineral salts it is the dissociated ion which is chiefly effective. It seemed quite possible that, if the effect of these reactions were taken into account, certain effects of salt antagonism might perhaps be explained without postulating any other than a quantitative difference between the various cations. It was to test this hypothesis that the present investigation was undertaken.

SYNTHETIC MEDIUM USED IN THE INVESTIGATION

In order to study this problem it was essential to obtain a medium of as simple a composition as possible, so that its components might not seriously interfere with the effect of the added cations either by precipitation, change in hydrogen ion concentration or contribution of other cations. In most of the earlier studies on growth and biochemical activity it has been quite impossible to be certain in regard to these points.

The medium devised by Dolt (1908) containing 1 per cent asparagin and 0.2 per cent $(NH_4)_2HPO_4$ was tested, as were various of the amino-acid media described by Zunz and György (1916) but either precipitates occurred (as a result of the high concentration of phosphate) or growth was too scanty for our pur-

poses. At last, after much experimentation, a new medium was devised (described by Dolloff (1927)) which proved very satisfactory. Its composition was as follows:

Ammonium tartrate $(NH_4)_2C_4H_4O_6$	5	grams
Lactose	5	grams
Ammonium phosphate $(NH_4)_2HPO_4$	0.02	grams
Distilled water	1000	cc.

This medium after autoclaving for fifteen to twenty minutes at 15 pounds pressure had a pH value of 4.9 to 5.0 and if care was taken to keep the pressure down to 15 pounds the sugar was unaffected and the medium remained colorless. The ammonium tartrate was recrystallized and the lactose was a Pfanstiehl c.p. special product, represented as free from sulphates, chlorides, aluminum, calcium and heavy metals.

Potassium phosphate, K_2HPO_4, could be substituted for ammonium phosphate; and in both the potassium and the ammonium media our strain of *Bact. coli* was carried for 81 transfers made at one to three-day intervals. Growth was just about as good in these media (as evidenced by turbidity) as in the pepton medium of Hotchkiss (1923).

The continued growth of bacteria for over 80 transfers in a medium practically free from any cation except ammonium is of considerable theoretical interest. Jordan (1899) reports the growth of *Ps. pyocyanea* in a medium containing asparagin alone but no long-continued cultivation was attempted.

One drawback of the Dolloff medium lay in the fact that it precipitated calcium and so could not be used for experiments with that salt. Ammonium oxalate proved an available substitute for ammonium tartrate as a source of nitrogen but the ammonium salts of formic acetic and lactic acid did not. In other words, when carbon was supplied in the form of lactose, nitrogen could be obtained from di-ammonium salts but not from mono-ammonium salts. This is in accord with the work of Koser (1923) who found that *Bact. coli* could obtain its nitrogen from di-ammonium phosphate; but could not utilize sodium oxalate or sodium tartrate as sources of carbon, requiring sodium acetate or lactate for this purpose. Apparently, with this

organism, a di-ammonium salt must supply nitrogen and a mono-ammonium salt carbon. It may be recalled, however, that Sullivan (1905) reported good growth of pigment-forming bacteria with ammonium lactate as the only source of nitrogen.

In our preliminary studies two other media were used, the 1 per cent pepton solution employed by Hotchkiss and a modified Dolt medium with the phosphate content reduced and lactose added. Its composition was as follows:

Asparagin	1 gram
Ammonium phosphate $(NH_4)_2HPO_4$	0.002 gram
Lactose	0.5 gram
Water	100 cc.

Our culture was carried in this medium for 34 transfers but growth was somewhat scanty.

The major part of our work (and all of it in which the use of asparagin or pepton media is not specifically noted) was done with the ammonium tartrate, lactose, ammonium phosphate medium described above. The medium was made up in double strength and mixed in large test tubes of hard glass with an appropriate volume of the salt solution to be studied, plus distilled water to make up the final concentration desired. The pH was unadjusted but was always determined and recorded. After sterilization of the resulting mixture the tubes were inoculated with the test organism. This was the same strain of *Bact. coli* (*communis*) used for many studies of viability in this laboratory by Winslow and Cohen, Winslow and Falk, Winslow and Shaughnessy and Hotchkiss. It was transferred from a stock agar slant by a straight wire to the salt-free tartrate-lactose medium and after incubation for twenty-four hours at 37 degrees in this medium 1 drop of the lactose-tartrate culture was added to the actual test solution with a pipette. This gave an initial seeding of 1 to 2 million bacteria per cubic centimeter. After eighteen to twenty hours incubation at 37 degrees the number present was determined by plating on agar. In the tartrate medium without salts the count after such a period would rise to 100 million. In the presence of a stimulating salt concentration it might rise to 500 million; and in the presence of a toxic salt concentration

it might fall to less than 10,000. In addition to the observations made in this way by plate counts (on which our main conclusions will be based) many other tests were run in which the growth of the bacteria was roughly estimated by observations of turbidity, the procedure followed by Hotchkiss.

All mineral salts used were Baker c.p. analyzed products and all glassware was stored in dichromate cleaning fluid, washed with hot water, rinsed three times in distilled water and drained dry.

STIMULATING AND TOXIC EFFECTS OF SINGLE SALTS IN VARIOUS MEDIA

It will, first of all, be of some interest to compare the general results obtained in regard to the stimulating and toxic effects of the individual salts with those reported in earlier papers from this laboratory. The data obtained by Winslow and Falk for Na and Ca in simple aqueous suspension, by Holland for K and Mg in aqueous suspension, by Hotchkiss for seven different salts in a pepton medium and by ourselves for the same seven salts in the tartrate or asparagin synthetic media or in pepton medium, are presented for comparison in table 1. These data were obtained for the most part by turbidity readings, checked occasionally by plate counts. All data subsequently cited are based on plate counts.

On the whole, the results check remarkably well. The only marked discrepancy appears in the case of $HgCl_2$ which shows much lower critical concentrations in our work than in that of Miss Hotchkiss.

In this preliminary study, one point was brought out which deserves special mention. We noted that the salt concentration showing stimulation differed somewhat according to the growth period which had elapsed at the time of observation. The more dilute concentrations showed stimulation first (say after twenty-four hours); while after forty-eight hours the next higher concentration would exhibit as much or more growth than the one which had been optimum at first. We found also that successive transfers in the tartrate medium containing 0.5 M NaCl or KCl

TABLE 1

Critical concentrations of various salts as determined by different investigators in various media

SALT	MEDIUM	OBSERVER	STIMULATING CONCENTRATION	SLIGHTLY TOXIC CONCENTRATION	HIGHLY TOXIC CONCENTRATION
			M	*M*	*M*
NaCl	Water	Winslow and Falk	0.014–0.145	0.43	0.7
	Tartrate	Winslow and Dolloff	0.025–0.300	0.40	0.6–0.7
	Pepton	Hotchkiss	0.025–0.250	0.50	2.0
KCl	Water	Holland	0.145–0.436		0.7
	Tartrate	Winslow and Dolloff	0.050–0.400	0.50	0.8
	Pepton	Winslow and Dolloff	0.250	0.75	1.0
	Pepton	Hotchkiss	0.250	0.50	2.0
$CaCl_2$	Water	Winslow and Falk	0.001–0.140		0.4
	Pepton	Winslow and Dolloff	0.050–0.070	0.10	0.2
	Pepton	Hotchkiss	0.050–0.250		0.5
$MgCl_2$	Water	Holland	0.020–0.080		0.5
	Tartrate	Winslow and Dolloff	0.003–0.070	0.10	0.2
	Pepton	Hotchkiss	0.050–0.100		0.5
$BaCl_2$	Asparagin	Winslow and Dolloff		0.10	0.2
	Pepton	Hotchkiss	0.050	0.10	0.2
$PbCl_2$	Asparagin	Winslow and Dolloff	0.0000001	0.0001	0.0005
	Pepton	Hotchkiss	0.00005		0.0005
$HgCl_2$	Tartrate	Winslow and Dolloff	0.00000001–0.00000005		
	Pepton	Winslow and Dolloff	0.00000001	0.00000005	0.0000005
	Pepton	Hotchkiss	0.000001	0.000005	0.00001

(a concentration slightly toxic to the bacteria fresh from a salt-free medium) led to an adaptation producing quite vigorous growth.

QUANTITATIVE EFFECTS OF Na, K AND Mg MEASURED IN TERMS OF DISSOCIATED IONS

It is noticeable in reviewing the literature dealing with antagonism that experimenters have given scant attention to the possible effects on the dissociation of salts, resulting from the simultaneous presence of other salts. Concentrations have in all cases been expressed in terms of molality or of iso-tonicity and in most instances the results have been judged by comparison of the effect of the mixture with that of a solution containing either salt alone, equal in molality or iso-tonicity as the case might be, to the sum of the two in the mixture. Since, however, neither molality nor iso-tonicity but concentration of dissociated ions is the primary measure of the influence of a salt mixture upon the bacterial cell, such a procedure seems illogical.

In our work, therefore, solutions of NaCl, KCl and $MgCl_2$[2] were made up in decimal molar concentrations and the ionic activities of the final pure solutions or mixtures were computed by means of the activity coefficients of Lewis and Randall (1923), using, in the case of binary solutions, their formula:

$$u = \frac{mv^2 \text{ for each ion}}{2}$$

where u = ionic strength of the solution, m = stoichiometric molality, and v = valence.

In computing these ionic activities it was necessary in the case of strong magnesium solutions to extrapolate beyond the range of the Lewis and Randall tables. This was done by the use of the curve presented in figure 1 and the values obtained by extrapolation are separated in our tables by a horizontal line from those directly calculated from the figures of Lewis and Randall. The concordance of the results obtained would suggest that our procedure was justified.

[2] We greatly desired to include $CaCl_2$ as well but were unable to find a suitable synthetic medium in which Ca would not precipitate.

Results as regards the action of the three pure salts studied are presented in table 2. In this and all succeeding tables each bacterial count represents the average of 4 to 6 determinations.

The results of these tests on the pure salts are graphically presented in figure 2. The bacterial numbers (in percentages of the number present in the salt-free tartrate medium) are plotted as ordinates, and the ionic activities as abscissae. It will be noted that the curves for Na and K are almost identical, showing definite stimulation at ionic activities between 0.05 and 0.2 with marked toxicity beyond 0.3. The curve for Mg, shown as a solid line, is of course very different on account of the greater

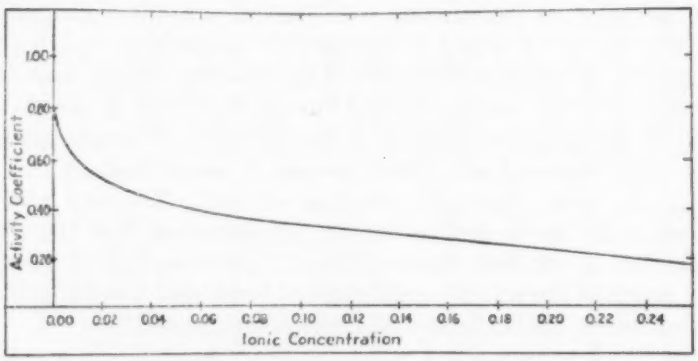

FIG. 1. CURVE FOR EXTRAPOLATION OF ACTIVITY COEFFICIENTS FOR COMPUTATION OF IONIC ACTIVITIES OF Mg^{++} AT IONIC CONCENTRATIONS GREATER THAN 0.1

activity of this cation. In order to test the theory that differences between the cations are quantitative rather than qualitative in nature, we re-plotted the Mg curve multiplying the ionic activity at each point by various constants. The ratio 8:1 gave most promising results; and the broken curve in the lower part of figure 2 is plotted from the actual ionic activities of Mg employed multiplied by 8.

It appears that the assumption that Mg is eight times as active as Na or K in its influence on bacterial viability gives a curve very similar to those obtained for the monovalent salts. It is

practically identical on the toxic side and shows stimulation in the same range on the stimulating side. The curve of stimulation

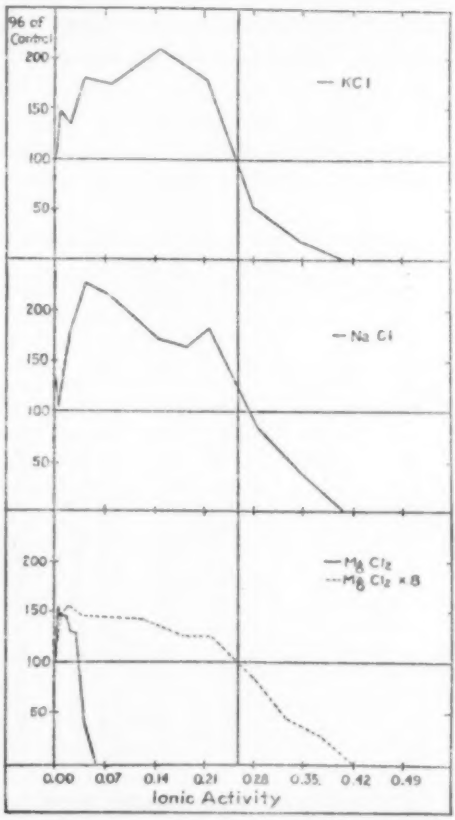

FIG. 2. VIABILITY OF BACTERIA IN SOLUTIONS OF SINGLE CATIONS OF VARYING STRENGTH

Abscissae represent computed ionic activities; ordinates represent the number of bacteria present expressed as a percentage of the number present in a salt-free control.

does not rise quite so high however and it may well be that the ratio of the stimulating effect of two salts is not necessarily exactly the same as that of the toxic effect of the same salts.

The results were, however, sufficiently close to encourage us to proceed with a study of the effects of K, Na and Mg in mutual combinations in order to see whether the assumption that Mg is eight times as active as either of the monovalent salts, but active in the same general manner, would explain the phenomena observed.

TABLE 2

Viability of Bact. coli in the presence of various ionic concentrations of K, Na and Mg

pH 4.6 to 5.2. Incubation period, 18 to 20 hours

K			Na			Mg		
Molality	Ionic activity	Bacteria per cubic centimeter	Molality	Ionic activity	Bacteria per cubic centimeter	Molality	Ionic activity	Bacteria per cubic centimeter
		millions			*millions*			*millions*
0	0	104	0	0	54	0	0	103
0.005	0.005	141	0.0001		62	0.0006	0.00049	136
0.010	0.009	156	0.001	0.001	72	0.0012	0.00093	149
0.025	0.022	143	0.005	0.005	58	0.0030	0.0022	159
0.05	0.042	187	0.025	0.022	97	0.0065	0.0042	151
0.1	0.079	183	0.05	0.042	123	0.012	0.0078	149
0.2	0.150	230	0.10	0.080	115	0.030	0.0149	147
0.3	0.219	178	0.20	0.150	93	0.051	0.0218	131
0.4	0.280	55	0.25	0.185	88	0.072	0.0280	128
0.5	0.340	24	0.3	0.219	99	0.10	0.0340	92
0.6	0.408	1.1	0.4	0.284	47	0.13	0.0407	47
0.7	0.469	0.3	0.5	0.344	23	0.17	0.0470	25
0.8	0.532	0.2	0.6	0.409	0.23	0.22	0.0528	0.29
0.9	0.594	0.2	0.7	0.471	0.18	0.27	0.0594	0.13
1.0	0.630	0.1	0.8	0.533	0.12	0.33	0.0632	0.24
			1.0	0.650	0.02	0.40	0.0640	0.28

EFFECT OF K, Na AND Mg IN COMBINATION, WITH CONSTANT SALT RATIOS BUT VARYING TOTAL MOLALITY

We next proceeded to test the effect of mixtures of K with Mg and of Na with Mg in which the ratio of the bivalent ion was kept at one tenth that of the monovalent ion, the total molality being varied as in the tests with the pure salts. The results are presented in tables 3 and 4 and in figure 3. The total ionic activity in the fifth column of the tables,—the value used in plotting the

figure,—is the ionic activity of Mg multiplied by eight, plus the ionic activity of the monovalent ion.

The curves for binary mixtures of Mg with either of the monovalent ions, if plotted directly against total ionic activity, are intermediate between the curves for the pure salts; but when the ionic activity of Mg is multiplied by eight as in figure 3 they become essentially the same as those for the pure salts presented in figure 2. Here, we are dealing with just the sort of mixture of

TABLE 3

Growth and viability of Bact. coli at various ionic activities of potassium plus magnesium in constant proportions

pH 4.8 to 5.0. Incubation period, 18 to 20 hours

KCl plus $MgCl_2$ molality		Ionic activity				Bacteria per cubic centimeter
		K	Mg	K plus Mg	K plus (Mg × 8)	
						million
0	0					101
0.00065	0.00007			0.00007		157
0.003	0.00036	0.00287	0.000279	0.0031	0.0051	144
0.012	0.0016	0.108	0.00104	0.012	0.0191	168
0.024	0.0035	0.021	0.0021	0.023	0.0378	183
0.045	0.007	0.037	0.0037	0.041	0.0666	156
0.100	0.016	0.077	0.0069	0.084	0.132	170
0.145	0.027	0.108	0.0104	0.118	0.191	153
0.20	0.040	0.144	0.0134	0.157	0.251	119
0.24	0.050	0.170	0.0155	0.185	0.294	85
0.30	0.065	0.205	0.0182	0.223	0.351	43
0.36	0.085	0.242	0.0212	0.263	0.412	5.7
0.45	0.115	0.294				0.25

monovalent and bivalent salts (ten parts of the former to one of the latter) described as antagonistic by earlier workers. From 5 parts to 20 parts of a monovalent salt to 1 part of a bivalent salt is the proportion usually reported as antagonistic. Yet no antagonism appears. In the more dilute solutions the favorable effects of the two salts are additive and in the higher concentrations the toxic effects are additive; while in the middle range (where one salt is favorable and the other toxic) the mixture gives

an intermediate effect. The phenomena seem best explained by the assumption that the dissociated ions act quite independently, the bivalent ion being some eight times more active than the univalent one.

EFFECT OF K, Na AND Mg IN COMBINATION, WITH CONSTANT MOLALITY BUT VARYING SALT RATIO

We next proceeded with an experiment along the line more commonly followed in studies of antagonism, by the use of salt

TABLE 4

Growth and viability of Bact. coli at various ionic activities of sodium plus magnesium in constant proportions

pH 4.8 to 5.0. Incubation period, 18 to 20 hours

NaCl plus $MgCl_2$ molality		Ionic activity				Bacteria per cubic centimeter
		Na	Mg	Na plus Mg	Na plus (Mg × 8)	
						million
0	0					117
0.00065	0.00007			0.00007		123
0.03	0.00036	0.00287	0.000279	0.0031	0.0051	155
0.012	0.0016	0.0108	0.00104	0.012	0.0191	190
0.024	0.0035	0.021	0.0021	0.023	0.0378	154
0.045	0.007	0.037	0.0037	0.041	0.0666	150
0.100	0.016	0.077	0.0069	0.084	0.132	193
0.145	0.027	0.108	0.0104	0.118	0.191	184
0.20	0.040	0.144	0.0134	0.157	0.251	138
0.24	0.050	0.170	0.0155	0.185	0.294	86
0.30	0.065	0.205	0.0182	0.223	0.351	39
0.36	0.085	0.242	0.0212	0.263	0.412	0.39
0.45	0.115	0.294				0.36

mixtures of constant total molality but with varying proportions of the salts in question, $MgCl_2$ being combined with NaCl and with KCl at total molalities of 0.1 and 0.5 in each case.

The results are presented in tables 5 and 6.

Inspection of the upper halves of tables 5 and 6 shows the type of evidence often cited as exhibiting antagonism. Thus, with K and Mg in a 0.1 M solution (upper half of table 5) KCl alone

is slightly favorable and $MgCl_2$ alone distinctly toxic but in intermediate mixtures the salts are highly favorable, the counts rising to a maximum at 1 part of Mg to 99 of K. With Na and Mg in 0.1 M solution, (upper half of table 6) one part of Mg to 99 of Na gives a maximum count, with a curve decreasing on each side.

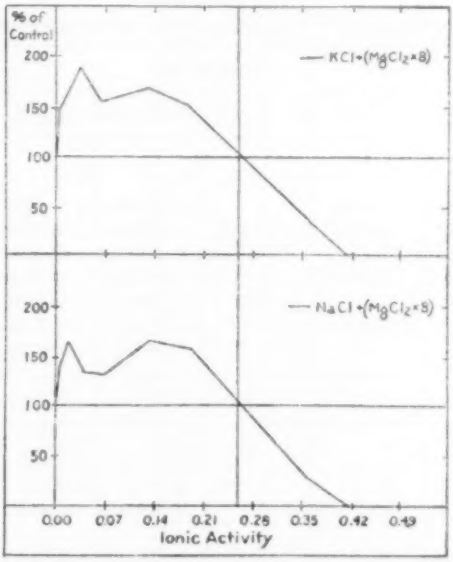

FIG. 3. VIABILITY OF BACTERIA IN PRESENCE OF A MONOVALENT AND A BIVALENT CATION WITH CONSTANT SALT RATIO AND VARYING TOTAL MOLALITY

Abscissae represent computed ionic activities (Mg being multiplied by 8); ordinates represent the number of bacteria present expressed as a percentage of the number present in a salt-free control.

Yet when we plot these data, as in the left half of figure 4, with the ionic concentration of Mg multiplied by eight, we have a curve identical with figures 2 and 3. The peak is due simply to an additive favorable effect of the two cations present.

On the other hand, the lower sections of tables 5 and 6 and the right half of figure 4 do suggest something like real antagonism, since in the toxic zone (0.5 M total concentration) a ratio of 1

part of Mg to about 60 parts of K and a ratio of 1 part of Mg to 20 parts of Na give a slightly lower toxicity than the pure monovalent salt. The antagonistic effect is, however, slight, in rela-

TABLE 5

Growth of Bact. coli at various ionic activities of potassium plus magnesium in varying proportions

pH 4.8. to 5.1. Incubation period, 18 to 20 hours

KCl plus $MgCl_2$		Ionic activity				Bacteria per cubic centimeter
		K	Mg	K plus Mg	K plus (Mg × 8)	
						million
0	0	0	0	0	0	154
0.100	0	0.0790	0	0.07902	0.0790	172
0.099	0.0010	0.0785	0.00052	0.07902	0.0826	229
0.098	0.0017	0.078	0.00082	0.07882	0.0846	208
0.095	0.005	0.075	0.00259	0.07759	0.0957	197
0.090	0.010	0.071	0.0046	0.0756	0.1080	188
0.083	0.017	0.065	0.0070	0.0720	0.1210	185
0.080	0.020	0.062	0.0088	0.0709	0.1325	179
0.075	0.025	0.059	0.0110	0.0700	0.147	171
0.067	0.033	0.051	0.0139	0.0651	0.162	177
0.050	0.05	0.045	0.020	0.0646	0.205	164
0	0.1	0	0.034	0.034	0.272	118
0	0	0	0	0	0	163
0.5000	0	0.340	0	0.3400	0.340	40
0.4950	0.0050	0.337	0.0014	0.3384	0.348	42
0.4917	0.0083	0.334	0.0023	0.3363	0.352	54
0.4750	0.025	0.322	0.0067	0.3287	0.376	48
0.4500	0.050	0.302	0.0125	0.3145	0.402	38
0.4170	0.083	0.278	0.0200	0.2980	0.438	4.2
0.400	0.100	0.265	0.024	0.289	0.457	0.08
0.375	0.125					0.08
0.33	0.17					0.15
0.25	0.25					0.49
0	0.5					0.26

tion to the general slope of the curve, which is mainly an expression of additive effects.

Since K and Na in pure solution give such similar curves no antagonism between these ions would be expected; but we made

series of tests in the toxic zone, the mildly stimulating zone and the highly stimulating zone, all of which showed that results were essentially the same, whatever the ratio of the two salts present.

TABLE 6

Growth of Bact. coli at various ionic activities of sodium plus magnesium in varying proportions

pH 4.8 to 5.0. Incubation period, 18 to 20 hours

NaCl plus $MgCl_2$		Ionic activity				Bacteria per cubic centimeter
		Na	Mg	Na plus Mg	Na plus (Mg × 8)	
						million
0	0	0	0	0		87
0.100	0	0.0800	0	0.08000	0.0800	150
0.099	0.0010	0.0790	0.00048	0.07948	0.0825	178
0.098	0.0017	0.0784	0.00082	0.07922	0.0850	159
0.095	0.005	0.0753	0.00235	0.07765	0.0941	153
0.090	0.010	0.0710	0.00460	0.07560	0.108	159
0.083	0.017	0.0649	0.00765	0.07255	0.126	155
0.080	0.020	0.0624	0.00880	0.07120	0.133	155
0.075	0.025	0.0518	0.01075	0.06885	0.138	144
0.067	0.033	0.0515	0.01286	0.06536	0.162	143
0.05	0.05	0.0376	0.021	0.0586	0.206	125
0	0.1	0	0.034	0.034	0.272	58
0	0	0	0	0		113
0.5000	0	0.344	0	0.3445	0.3445	13
0.4950	0.005	0.340	0.0014	0.3414	0.3512	15
0.4917	0.008	0.338	0.0023	0.3403	0.3564	9
0.475	0.025	0.325	0.0067	0.3317	0.379	16
0.45	0.050	0.306	0.0125	0.3185	0.406	0.63
0.47	0.083	0.281	0.0200	0.301	0.441	0.73
0.40	0.100	0.269	0.0240	0.293	0.461	0.58
0.37	0.125					0.61
0.33	0.17					0.49
0.25	0.25					0.40
0	0.5					0.14

EFFECT OF HYDROGEN ION CONCENTRATION UPON THE ACTIVITY OF K

Finally, we have conducted one experiment to test the effect of the reaction of the medium upon the activity of KCl. All of

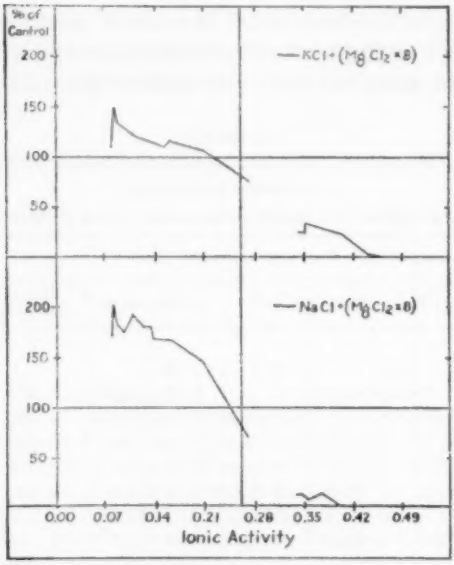

FIG. 4. VIABILITY OF BACTERIA IN PRESENCE OF A MONOVALENT AND A BIVALENT CATION WITH CONSTANT TOTAL MOLALITIES AND VARYING SALT RATIOS

Abscissae represent computed ionic activities (Mg being multiplied by 8); ordinates represent the number of bacteria present expressed as a percentage of the number present in a salt-free control.

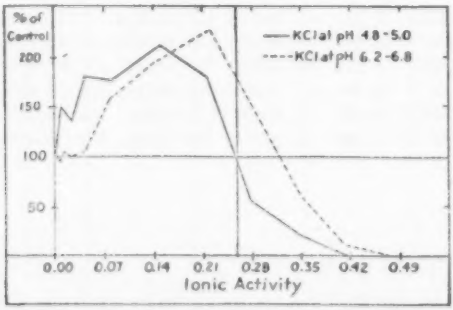

FIG. 5. VIABILITY OF BACTERIA IN POTASSIUM CHLORIDE SOLUTIONS OF VARYING STRENGTH AND AT TWO DIFFERENT HYDROGEN ION CONCENTRATIONS

Abscissae represent computed ionic activities; ordinates represent the number of bacteria present expressed as a percentage of the number present in a salt-free control.

our work so far reported was, as we have pointed out, conducted at a constant pH varying between 4.8 and 5.1 (with Mg it went down to 4.6). For comparison with the basic KCl curve (the data for which are presented in table 2) we ran a test in the usual tartrate medium but with its pH adjusted to 6.2 to 6.8 by the addition of NH_4OH. The results are presented in table 7 and are plotted for comparison with those obtained at pH 4.8 to 5.0 in figure 5.

TABLE 7

Growth and viability of Bact. coli at various ionic activities of potassium

pH 6.2 to 6.8. Incubation period, 18 to 20 hours

KCl MOLALITY	IONIC ACTIVITY	BACTERIA PER CUBIC CENTIMETER
		million
0	0	104
0.005	0.00475	141
0.010	0.0092	156
0.025	0.022	143
0.05	0.042	187
0.1	0.079	183
0.2	0.160	230
0.3	0.219	178
0.4	0.280	55
0.5	0.340	24
0.6	0.408	1.1
0.7	0.469	0.3
0.8	0.532	0.2
0.9	0.594	0.2
M	0.63	0.1

It seems almost incredible that the influence of hydrogen should differ from that of other cations only in degree; yet the curves as presented strongly suggest such a conclusion. In the stimulating range the acid solution is more stimulating than the alkaline one and in the toxic range it is more toxic,—exactly as if the H ion in each range operated in the same way as the K ion. The two curves are furthermore almost equally distant from each other where they touch the base lines and where they cross from the stimulating to the toxic zone. A computation of the quantitative efficiency of the H ion based on these intervals (disregarding the

effect of the NH_4 ion with which it was replaced) would suggest that H is some 30,000 times more active than K but that its effect is essentially of the same general nature. Much more work would of course be necessary before this suggestion can be substantiated.

GENERAL CONCLUSIONS

1. We have confirmed the findings of Hotchkiss and other earlier investigators to the effect that a wide variety of metallic cations (including in our study sodium, potassium, calcium, magnesium, barium, lead and mercury) stimulate the growth of bacteria when present in sufficiently low concentration and inhibit it when present in sufficiently high concentration, both the stimulating and the toxic concentrations varying widely for the different salts.

2. Our more detailed studies of the influence of potassium, sodium and magnesium upon the viability of *Bact. coli* in a simple synthetic medium indicate that the effect of these salts is qualitatively identical, magnesium being quantitatively about eight times as effective in a given concentration as either of the monovalent ions. The effect of the bivalent salt is apparently, however, slightly less in relation to the monovalent salts in the stimulating than in the toxic zone.

3. The effect of mixtures of magnesium with either sodium or potassium in the proportion of ten parts of the monovalent to one part of the bivalent ion in acid and neutral media is in the main a direct function of the ionic activity of the cations present and shows no evidence of antagonism.

4. A mixture of magnesium with either sodium or potassium, in varying proportions but with a constant molality, in the stimulating zone (0.1 M) shows essentially additive effects and yields curves identical with those obtained for the pure salts.

5. Similar studies in the toxic zone (0.5 M) show primarily additive effects with a slight suggestion of antagonism at ratios of 20 to 60 parts of the monovalent to the bivalent salt.

6. A single series of experiments on the effect of solutions of different hydrogen-ion concentration would seem to suggest that

even the hydrogen ion may play a rôle in its effect upon bacterial viability qualitatively the same as that of potassium, sodium and magnesium but with a coefficient of activity 30,000 times higher than that of the other univalent ions.

7. There is of course no reason to expect that all phenomena of salt action will fall under any single simple category. Particularly in the case of the higher plants and animals there must be many complex chemical as well as physical relationships involved. There is therefore no necessary conflict between our observations and those of other observers (except perhaps with the very peculiar results reported by Eisler (1909). It seems of interest, however, to point out certain broad principles which seem of significance in regard to the subject as a whole:

A. The cations exert a certain primary effect upon the bacterial cell (and possibly upon other plant and animal cells) which is qualitatively the same for all cations (perhaps even for hydrogen), but quantitatively different for each cation. The bivalent ions are eight or ten times as potent as the univalent ions in this regard. Such ions as mercury and hydrogen are several thousand fold more powerful. So far as viability is concerned, all cations appear to stimulate growth in a certain low concentration and to inhibit it in a certain higher concentration. The work of Shearer (1917, 1919), Shaughnessy and Winslow (1927) and others suggests that this action upon viability is related to an increased permeability due to dilute salts and a decreased permeability due to concentrated salts,—the latter followed by an increased permeability, of a different sort from that induced by dilute salts and probably due to rupture or some irreversible physical change in the cell membrane.

B. So far as the phenomena in question are concerned we can only postulate what may be termed *quantitative antagonism*. In the case of viability for example the mixture of concentrations of two salts, each of which alone was below the optimum ionic activity would show a stimulating effect. Thus, in the work of Lipman (1909) on ammonification varying proportions of calcium and of magnesium were mixed with solutions of potassium (all of 0.35 M strength). The observation that a mixture of the mono-

valent and bivalent salts in the proportion of about 10:1 was more favorable than either salt alone may have been due merely to the fact that the addition of calcium chloride or magnesium chloride increased the total ionic activity to an optimum value. Indeed Lipman's curves run almost parallel to our own, and we suspect that a more detailed series of experiments along this line would exhibit what we have called quantitative antagonism. Similarly, the results of Shearer (1917, 1919) are easily explicable on the same assumption. He found that 0.85 per cent sodium chloride was highly toxic for the meningococcus, and caused a marked decrease in electrical resistance in the suspension. This action was neutralized by a small addition of calcium chloride; but it was also abolished by increasing the concentration of sodium chloride. This indeed is the crucial test of "quantitative antagonism." If salt A produces a given effect and the addition of salt B abolishes it,—can we also abolish it by merely increasing the concentration of salt A? If so, we are dealing with quantitative antagonism,—really an additive effect.

C. There are unquestionably other cases of salt action, such as Neuschloss' studies of the effect of salts on the surface tension of lecithin and Osterhout's work on plant seedlings (1922), Ringer's fundamental studies of the control of muscle action by salts (1882), many of Loeb's results (1912), and the work of Brooks on carbon dioxide production (1920) which can only be explained on the assumption of specific and qualitatively different chemical reactions on the part of the various cations; and such reactions exhibit true *qualitative* antagonism. In many of these instances (as in the case of Osterhout's work on plant seedlings, Neuschloss' on lecithin and Brooks' on carbon dioxide production) it is evident that we are dealing with a phenomenon widely different from that of primary cation effects since in all these cases the bivalent and monovalent ions fail to show the marked difference in activity which they usually exhibit. On the other hand Osterhout's work on the electrical resistance of Laminaria seems to show a very close analogy to the primary effect of cations on bacterial permeability. One cannot help wondering whether the phenomena here revealed could not perhaps be explained on the basis

of quantitative antagonism rather than on Osterhout's assumption of a double salt, which of course implies a qualitative difference in the effect of the cations concerned. It does not seem impossible that some of Loeb's results with fish embryos might also be explained as due to quantitative antagonism.

D. So far as bacterial viability is concerned, the clearest evidence of qualitative salt antagonism is to be found in the earlier contributions from our own laboratory by Winslow and Falk (1918), Holland (1922) and Winslow and Falk (1923). In the last of these papers, however, it is shown, that the phenomena of antagonism appear only in a medium held at a pH of 7.0 or more. At more acid reactions the effects of sodium and calcium are additive and not antagonistic. The authors conclude that the harmful effects of calcium in the alkaline range are due, not to the primary toxicity of the salt but to its interference with the special reactions by which the bacterial cell neutralizes the alkalinity of the menstruum. The slight antagonism between potassium and magnesium demonstrated by Holland is susceptible of the same explanation, appearing only in an alkaline medium. From later work in this laboratory (Shaughnessy and Winslow 1927) it appears possible that the whole chain of phenomena in an alkaline medium may be in large part related to the effect of the salts upon the absorption of carbon dioxide from the atmosphere. Brooks' work on carbon dioxide production by bacteria (1920) may perhaps be affected by this phenomenon.

E. In view of the facts set forth we believe the evidence is sufficient to indicate the desirability of reconsidering all data in regard to salt antagonism in order to see how far the phenomena recorded in a given case can be explained on the assumption of a purely quantitative difference between the activity of the cations concerned,—so that a clear distinction may be drawn between "quantitative antagonism" which is really an additive reaction and true "qualitative antagonism," associated with specific differences between the chemical effects of the various ions. The latter appears to play a relatively unimportant part so far as the influence of the cations upon bacterial viability is concerned.

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A MODIFICATION OF THE ALCOHOL TEST AS AN INDEX OF THE BACTERIAL COUNT AND KEEPING QUALITY OF MILK

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During the past few years several simple methods have been devised by which it is claimed that the approximate bacterial count of milk can be estimated. The most successful of these is the Reductase-Fermentation Test of Barthel and Jensen. Hiscox and Starling (1925), however, have shown that this test "does not afford a satisfactory method for differentiating between samples having a low bacterial count."

A rapid method whereby the approximate bacterial content of a sample of milk containing relatively few organisms could be estimated would be appreciated alike by condenseries, creameries, town and village health departments. Such a test, however, would require to be simple and effective; that is (1) it should not require the services of a trained technician, (2) it should show not only the activities of bacteria but also of enzymes present, (3) it should be capable of being performed rapidly, (4) it should furnish the results in a short time and (5) it should entail the minimum of expense per sample.

It is improbable, however, that any single test can be evolved which would fulfil all these conditions and at the same time be reliable.

Since the only satisfactory basis, from a commercial point of view, upon which to judge the cleanliness of a milk, is by the time which elapses before it actually sours, this question must receive primary consideration. Milk may be of high food value and free from pathogenic bacteria, but if it sours within a few hours of delivery, it is of little use. The keeping quality of milk is inti-

mately bound up with bacterial and enzymatic activities and hence the keeping quality is a rough guide to the conditions under which the milk has been produced and also to its bacterial count.

A more satisfactory and scientific method of estimating the keeping quality is required and it was hoped that a modification of the Alcohol Test would meet this need and at the same time provide a better means of determining the approximate bacterial count of milk samples.

The Alcohol Test first came into use in 1890 and has since, to some extent, been employed both in Europe and in America for grading milk in the receiving room. The test consists of adding 2 cc. of alcohol to 2 cc. of milk and shaking this in a test tube, the strength of the alcohol used being generally 68 to 70 per cent by volume. If a precipitate is formed the test is said to be positive and such milk is held to be of poor hygienic quality.

The precipitation of the casein by the alcohol appears to be intimately associated with (1) the degree of acidity of the milk (2) the amount of rennet present and (3) the balance of the milk salts. The greater the amount of acid or rennet present, the more readily will the casein be precipitated.

Abnormal milks such as colostrum, milk from diseased udders and from diseased cows, also produce a precipitate with alcohol. Such a precipitate, however, appears dependent on the proportions of the different salts in the milk. Sommer and Hart (1921) and Sommer and Binney (1923) have shown that "the calcium and magnesium, and the citrates and phosphates, present in milk have a very marked effect on both the heat coagulation and the alcohol coagulation of milk." It has also been proved by the same workers "that there is a balancing effect between the calcium and magnesium on the one hand and the citrates and phosphates on the other, the proper balance of these producing the most stable condition, an excess of either causing milk to coagulate more readily." It is evident that such premature curdling may easily occur in milks which neither show an abnormally high bacterial count nor yet a poor keeping quality when judged by ordinary methods. This does not, however, detract from the value of the alcohol test or any modification thereof since in such cases an abnormal condition of the milk is evident.

Although the present alcohol test does indicate the presence of acid and rennet—the principal end products of the more common organisms affecting the keeping quality of milk—it does not however, provide any information regarding the degree of concentration of acid or rennet present. Hence the necessity for a modification of the test. Such a modification should show different degrees of acidity so that the keeping quality could be more accurately estimated and its relation to the bacterial count more fully established.

The modification of the present alcohol test now suggested involves (*a*) incubation of the milk for a definite period at a specified temperature before the addition of the alcohol, (*b*) an alteration in the concentration of the alcohol employed and (*c*) the addition of an indicator to the alcohol.

The incubation of the milk is necessary in order that sufficient acid and rennet may be developed within the milk before the alcohol is added. This treatment of the milk no doubt complicates the test to a slight extent but experience has shown it to be necessary in order to obtain more accurate results in differentiating between milks of varying bacterial count and keeping quality—lack of accuracy in this respect having been proved to be one of the weakest points in the original test.

An increase in the concentration of the alcohol is recommended, as with milks of low bacterial count it has been found that the small amount of rennet developed, even after incubation, is insufficient to give a precipitate with the strength of alcohol used in the original test.

The addition of the indicator to the alcohol provides a means of determining approximately the degree of acidity developed in the milk.

In order to obtain definite information as to the most suitable modifications on the above lines the following points required investigation: (*a*) The optimum temperature of incubation; (*b*) the minimum period of incubation of the milk before testing; (*c*) the most suitable concentration of alcohol; (*d*) the most reliable indicator.

EXPERIMENTAL

In order to obtain information on these points the rate of development of acidity in milk at different temperatures was first studied.

Thirty samples of milk were incubated at both 37° and 30°C. and each titrated after two, four, six, eight and twenty-two hours' incubation.

To obviate the necessity of interfering with the bulk of the sample at every test, each was divided into ten portions of 40 cc. Five of these portions were incubated at 37°C. and the remaining five at 30°C.

Tests were made on portions from both incubators at the same time, one portion from each sample being used for each test.

The milk was titrated against N/40 NaOH using phenol phthalein as an indicator, as the figure thus obtained gave the number of degrees of acidity according to the Soxhlet-Henkel scale. This was converted to a percentage of acid equivalent to lactic acid, by multiplying by 0.0218.

Table 1 shows the rate of development of acidity at 37° and 30°C. at varying periods in the thirty samples of milk examined.

On examination of these results it will be seen that the milks can be divided into four classes as given in table 2. Those samples marked with one star (*) have been averaged and compose Class 1, those with two stars (**) Class 2, those with one cross (+) Class 3 and those with two crosses (++) Class 4.

The average rate of development of acidity in the samples incubated at 37°C. in each of these classes is illustrated in figure 1.

These results show that when different samples of milk are held at a constant temperature their rate of development of acidity varies considerably. Some of the milks, viz., those in Class 2, showed a decrease in acidity during the first four to six hours which was followed by a rapid increase, while the samples in Class 1 which also showed a decrease in acidity during the first few hours developed little acidity even after a period of twenty-two hours' incubation at 37°C. In the case of those milks in Classes 3 and 4 there was a steady increase in acidity after the first two hours' incubation.

TABLE 1

Development of acidity in milk at 37° and 30°C.

NUMBER OF SAMPLE	INITIAL ACIDITY (PER CENT) (= LACTIC ACID)	PERCENTAGE ACIDITY (= LACTIC ACID) DEVELOPED AFTER				
		2 hours	4 hours	6 hours	8 hours	22 hours
Temperature of Incubation 37°C. Average age of the samples when test was commenced, 17½ hours						
* 1	0.16350	0.16350	0.15914	0.16350	0.16568	0.47960
+ 2	0.19620	0.19620	0.20056	0.22236	0.3052	0.7630
** 3	0.18530	0.18530	0.18530	0.19184	0.20056	0.74120
+ 4	0.18966	0.18966	0.19402	0.20928	0.28122	0.74120
* 5	0.17222	0.17000	0.16786	0.17004	0.17440	0.44690
* 6	0.17222	0.17000	0.16568	0.16786	0.17222	0.52320
** 7	0.21582	0.21583	0.21146	0.22236	0.23108	0.74120
++ 8	0.20710	0.21364	0.25070	0.28558	0.35970	0.7848
** 9	0.20928	0.20928	0.20710	0.21582	0.24634	0.69760
** 10	0.18748	0.18748	0.18530	0.19402	0.20274	0.80660
** 11	0.20056	0.20056	0.18938	0.20928	0.21582	0.71940
* 12	0.17876	0.17658	0.17440	0.17876	0.17876	0.41856
++ 13	0.20492	0.21146	0.24416	0.27250	0.35752	0.7630
** 14	0.20274	0.20274	0.20274	0.20710	0.2180	0.7412
** 15	0.20056	0.19838	0.19838	0.20274	0.21364	0.7848
** 16	0.19184	0.19184	0.19402	0.20056	0.21146	0.7412
* 17	0.17658	0.17440	0.17222	0.17658	0.17876	0.45998
* 18	0.17440	0.17440	0.17004	0.17222	0.17658	0.44036
** 19	0.20710	0.20710	0.20492	0.20928	0.22454	0.7630
+ 20	0.19184	0.19184	0.20056	0.22672	0.29866	0.7848
+ 20	0.20056	0.20056	0.20492	0.22236	0.35316	0.7412
++ 22	0.20928	0.21582	0.25724	0.29866	0.39458	1.0028
* 23	0.17658	0.17440	0.17222	0.17658	0.17658	0.54500
** 24	0.20056	0.20056	0.19838	0.20492	0.21582	0.7412
+ 25	0.20274	0.20274	0.20710	0.23108	0.26160	0.8066
++ 26	0.20710	0.21364	0.26160	0.29648	0.3924	0.8066
** 27	0.19402	0.19402	0.19184	0.20056	0.23108	0.6976
** 28	0.19838	0.19838	0.19838	0.20274	0.21582	0.80660
++ 29	0.21582	0.22454	0.29866	0.32700	0.41420	0.87200
+ 30	0.20492	0.20492	0.20928	0.23544	0.28776	0.76300
Temperature of Incubation 30°C. Average age of the samples when test was commenced, 17½ hours						
* 1	0.16350	0.16350	0.16350	0.16132	0.16350	0.19838
+ 2	0.19620	0.19620	0.20056	0.22236	0.3052	0.7630
** 3	0.18530	0.18530	0.18530	0.18748	0.19184	0.65400
+ 4	0.18966	0.18966	0.19402	0.20928	0.28122	0.7412

TABLE 1—*Continued*

NUMBER OF SAMPLE	INITIAL ACIDITY (PER CENT) (= LACTIC ACID)	PERCENTAGE ACIDITY (= LACTIC ACID) DEVELOPED AFTER				
		2 hours	4 hours	6 hours	8 hours	22 hours
Temperature of Incubation, 30°C.—*Continued*						
* 5	0.17222	0.17222	0.17004	0.17004	0.17440	0.22454
* 6	0.17222	0.17222	0.17004	0.16786	0.17220	0.22672
** 7	0.21582	0.21582	0.21146	0.21364	0.22236	0.6540
++ 8	0.20710	0.20928	0.22454	0.23980	0.27468	0.74120
** 9	0.20928	0.20928	0.20710	0.20928	0.21582	0.6104
** 10	0.18748	0.18748	0.18530	0.18530	0.19402	0.69760
** 11	0.20056	0.20056	0.19838	0.19838	0.20928	0.6540
* 12	0.17876	0.17876	0.17658	0.17876	0.18094	0.22890
++ 13	0.20492	0.20710	0.21800	0.23762	0.27904	0.78480
** 14	0.00274	0.20274	0.20274	0.20492	0.20710	0.6104
** 15	0.20056	0.20056	0.19838	0.20274	0.20492	0.6104
** 16	0.19184	0.19184	0.19184	0.19402	0.19838	0.6540
* 17	0.17658	0.17658	0.17658	0.17440	0.17658	0.22672
* 18	0.17440	0.17440	0.17440	0.17658	0.17658	0.22454
** 19	0.20710	0.20710	0.20492	0.20710	0.21364	0.58860
+ 20	0.19184	0.19184	0.20056	0.22672	0.29866	0.7848
+ 21	0.20056	0.20056	0.20492	0.22236	0.35316	0.74120
++ 22	0.20928	0.21146	0.22890	0.24198	0.28340	0.8284
* 23	0.17658	0.17658	0.17658	0.17658	0.17658	0.23108
** 24	0.20056	0.20056	0.19838	0.20056	0.20234	0.58860
+ 25	0.20274	0.20274	0.20492	0.22236	0.25070	0.61040
++ 26	0.20710	0.20928	0.23544	0.24416	0.29648	0.7630
** 27	0.19402	0.19402	0.19184	0.19184	0.20056	0.6104
** 28	0.19838	0.19838	0.19838	0.20056	0.20274	0.6322
++ 29	0.21582	0.21800	0.27250	0.28776	0.30520	0.82840
+ 30	0.20492	0.20492	0.20928	0.23644	0.28776	0.7630

Although in the majority of the samples incubated at 37°C. acidity developed rapidly during the first few hours, it will be seen that in a few cases increase in the acidity was not noticeable until a period of at least eight hours had elapsed. In the case of those milks incubated at 30°C. very little acidity had developed even after incubation for eight hours. It is obvious therefore, that incubation of the milk at 37°C. is preferable and that eight hours must be regarded as the minimum length of time of incubation at this temperature.

In order to obtain some information as to the most suitable concentration of alcohol for carrying out the test, trials were made with various strengths of alcohol, at the same time that the acidity determinations were made. As a result of previous work on similar lines, concentrations of 73, 78 and 83 per cent by volume of alcohol were used for these trials. The tests were carried out in triplicate; two cubic centimetres of each concentration being added to similar quantities of the milk being tested. The acidity at which a precipitate occurred was found to vary greatly

TABLE 2

Development of acidity in milk held at 37° and 50°C.

MILK SAMPLES	TEMPERATURE	AVERAGE INITIAL ACIDITY PER CENT (= LACTIC ACID)	AVERAGE (PER CENT) ACIDITY (= LACTIC ACID) DEVELOPED AFTER				
			2 hours	4 hours	6 hours	8 hours	22 hours
	°C.						
Class 1: Nos. 1, 5, 6, 12, 17, 18, 23	37	0.17340	0.17189	0.16879	0.17222	0.17471	0.47337
	30	0.17346	0.17346	0.17253	0.17082	0.17439	0.22298
Class 2: Nos. 3, 7, 9, 10, 11, 14, 15, 16, 19, 24, 27, 28	37	0.19947	0.19928	0.19801	0.20510	0.21890	0.74846
	30	0.19947	0.19947	0.19783	0.19965	0.20528	0.63038
Class 3: Nos. 2, 4, 20, 21, 25, 30	37	0.19765	0.19765	0.20274	0.22454	0.29793	0.76663
	30	0.19765	0.19765	0.20237	0.22308	0.29612	0.7339
Class 4: Nos. 8, 13, 22, 26, 29	37	0.20884	0.21582	0.26247	0.29604	0.38368	0.84584
	30	0.20884	0.21062	0.23587	0.25026	0.28776	0.78916

—a variation due probably to the presence of rennet producing organisms. For example those milks in Class 1 which developed little acidity after eight hours' incubation at 37°C. gave a moderately heavy precipitate with 83 per cent Alcohol, while those in Class 2 which developed a greater acidity after the same period of incubation gave, at the most, a very fine precipitate. This difference in action of these two groups of milks is again attributed to the presence of rennet producing organisms.

The results obtained from the use of 73 and 78 per cent alcohol and an incubation temperature of 37°C. and with 73, 78 and 83

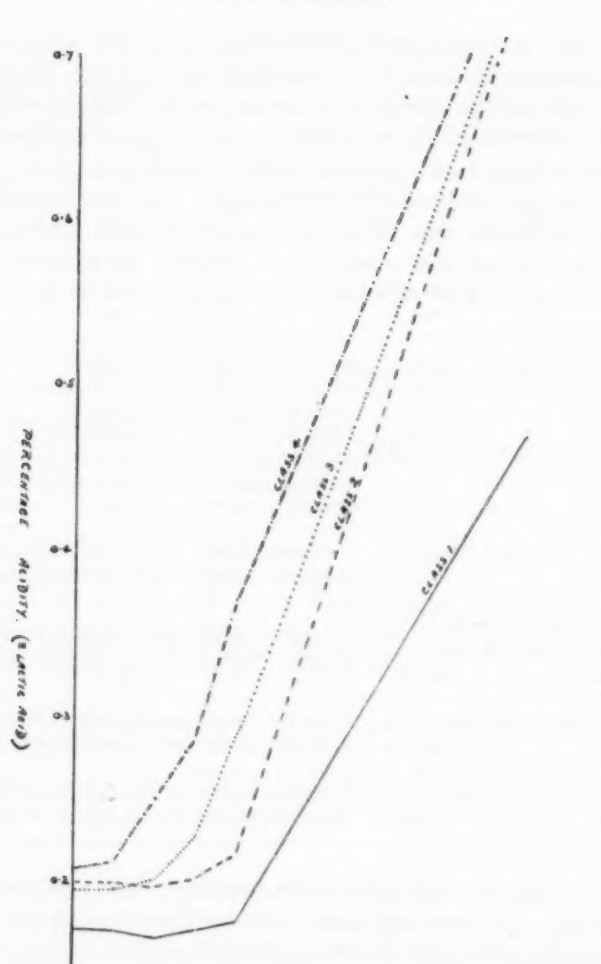

FIG 1. GRAPHS SHOWING THE AVERAGE RATE OF DEVELOPMENT OF ACIDITY IN MILKS HELD AT 37°C. IN CLASSES 1, 2, 3 AND 4

per cent Alcohol and an incubation temperature of 30°C. showed, that in the case of some milks the rennet produced during the incubation period was insufficient to be detected by these strengths of alcohol. It was concluded, therefore, that by incubation of the milk at 37°C. for a minimum period of eight hours and by the use of 83 per cent alcohol it was possible to detect milks which contained a larger number of rennet producing organisms than acid producing bacteria although the total count in each milk was relatively small.

Rice and Markley (1924) have shown that "The concentration of hydrogen ion and acidity by titration vary in the same direction quite regularly although not to the same extent." Hence some information as to the most suitable indicator for addition to the alcohol was obtainable from a study of the figures relating to the rate of development of acidity given in tables 1 and 2. From these tables it is seen that the greatest average acidity developed after eight hours' incubation at 37°C. is 0.373 per cent which represents a pH value in milk of approximately 4.5. The hydrogen ion concentration of fresh milk varies from 6.4 to 6.6 and therefore the pH range under consideration is approximately 4.5 to 6.4. Consequently, it would appear that the most suitable indicator for addition to the alcohol is either Brom Cresol Purple or Methyl Red. Both these indicators were used, being made up in 83 per cent alcohol (by volume) and using 0.02 per cent of the dye. Tests were then carried out on samples of milk which had been incubated at 37°C. for eight hours; in each case 2 cc. of the alcohol containing the indicator were added to 2 cc. of milk and the color and type of precipitate, if any, noted. It was found that the color changes given with Brom-Cresol Purple were not so easy to differentiate as those given by Methyl Red and that they could only with difficulty be matched with the color standards of Ridgeway (1912). Methyl Red is consequently recommended.

From these experiments, therefore, it was concluded that the following modifications in the alcohol test gave the best results: (1) A preliminary incubation period of at least eight hours at 37°C; (2) the use of 83 per cent (by volume) neutral alcohol containing 0.02 per cent Methyl Red as against the use of 70 per cent (by volume) alcohol only.

The Alcohol Test thus modified was applied to a large number of milks having a keeping quality range of from one-half to over three days and whose bacterial counts varied from 500 to over one million per cubic centimetre.

TABLE 3

COLOR ACCORDING TO RIDGEWAY (1912)	TYPE OF PRECIPITATE	APPROXIMATE TOTAL BACTERIAL COUNT PER CUBIC CENTIMETER*	APPROXIMATE KEEPING QUALITY IN DAYS (AT 60°F.) (I. E., FROM THE TIME THE TEST WAS COMMENCED UNTIL THE MILK CURDLED ON BOILING
Mikado Orange	Absent or very fine	Under 10,000	3 or over
	Moderately heavy	50,000	2½
	Very heavy	200,000	2
Salmon, Orange	Moderately heavy	50,000	2½
	Very heavy	200,000	2
Flame Scarlet	Moderately heavy	200,000	2
	Very heavy	500,000	1½
Peach Red	Heavy	500,000	1½
Scarlet to Scarlet Red	Heavy	Under 1 million	1
Spectrum Red (curdled)	Heavy	Over 1 million	½

* The total bacterial counts were determined on Nutrient medium having the following composition:

Lemco	0.3 per cent
Agar-Agar (Shred)	1.5 per cent
Peptone (Allen and Hanbury's)	0.5 per cent
Neutralized to pH	7.2

Forty cubic centimetres of a milk to be tested were poured into a sterile boiling tube fitted with a rubber stopper and incubated for nine[1] hours at 37°C. The milk was then cooled by immersion of the tube in water, shaken vigorously and 2 cc.

[1] As the result of many experiments it was found that more accurate results could be obtained by incubating the milk for nine hours in preference to eight hours.

poured into a clean dry test tube. To this were added 2 cc. of 83 per cent Methyl Red alcohol the color and type of the precipitate being noted and compared with the keeping quality. From the results of these tests Table 3 has been drawn up.

The cooling of the milk after incubation is important in order to obtain the correct color (Clark, 1925). Care must also be taken to ensure that the test tubes used are properly cleansed as the slightest trace of alkali or acid present may effect a considerable change in the final color obtained and lead to an erroneous interpretation of the results. Each test should preferably be carried out in duplicate.

To save time and trouble involved in measuring equal quantities of milk and alcohol by means of pipettes, the use of test tubes marked off at 2 and 4 cc. is suggested.

EXPLANATION OF TABLE 3

The color given under the heading "Color according to Ridgeway (1912)," is a guide to the degree of acidity produced in the milk, while the precipitate, in the absence of acid, indicates roughly the amount of rennet present. For example, a milk which a "Mikado Orange" and no precipitate will probably keep sweet for half a day longer than one which gives the same color and produces a moderately heavy precipitate.

A heavy precipitate which is very granular indicates a poorer keeping quality than one which though heavy is fine in texture.

It should be mentioned that the above tests have been carried out on milk which was from fifteen to twenty-seven hours old obtained from Shorthorn and Ayrshire cows. Since the fat globules of milk do not absorb the color of Methyl Red, milks high in fat such as those from Jersey and Guernsey cattle are liable to give slightly lighter colors.

CONCLUSIONS

The results of these experiments show that the Alcohol Test can be modified to give an index of the bacterial count and keeping quality of milk and consequently of its commercial value. It should be borne in mind, however, that the complexity and

number of the factors which affect the keeping quality of milk as judged by the consumer cannot all be taken into account by any one method. Certain abnormal flavors and odors, for example, can only be detected by taste and smell. Moreover, during the bactericidal period the results of the actions of bacteria and enzymes on the milk are too small to be measured, except by the most delicate tests. All one can hope to do, therefore, is to choose a method which approximates most closely to the ideal.

My thanks are due to Dr. James Ewing for carrying out pH determinations on many samples of milk by the electrometric method, and to Mr. A. Paton, B.Sc., N.D.A., N.D.D., for valuable help and criticism.

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THE RELATION BETWEEN ELECTRICAL CHARGE AND THE AGGLUTINATING ABILITY OF SALMONELLA PULLORUM[1,2]

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INTRODUCTION

Attention has been repeatedly called to variations in the agglutinability of strains within a bacterial species. This has been especially true of *Eberthella typhi*. Some strains of this species have been encountered which were agglutinated so poorly that they could not be used in the Widal test. On the other hand, the extensive study of Ishii (1922b) shows many hyper- or spontaneously agglutinated strains. Zinsser (1923) considers it a common observation that no two strains of a species are exactly similar in their agglutinability. The various reviews of bacterial agglutination published, especially those of Arkwright (1914); Tulloch (1914); Hooker (1916); Buchanan (1919); Ishii (1922 a and b); Morishima (1921) and Robinson (1923) make another summary of the literature superfluous. Therefore, reference will be made only to the literature which deals with *Salmonella pullorum*.

Extreme variations in the agglutinability of strains of *Salmonella pullorum*, as observed by the senior author, led to the investigation reported in this paper. Recently several investigators have reported similar observations. Mallman (1925) found that

[1] This is a résumé of a thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Master of Science in Agricultural and Biological Chemistry in the Graduate School of the Pennsylvania State College, 1926.

[2] Published by Permission of the Director of the Pennsylvania Experiment Station as Technical Paper 439.

strains vary in their ability to be agglutinated and questioned the accuracy of the agglutination test unless strains of consistently high titre are used. May and Goodner (1926), after finding considerable variations in agglutinability of strains, concluded that "it seems advisable to select highly agglutinating strains for the routine test for ovarian infection in adult fowls." Gwatkin (1925) has also found that all strains are not agglutinated equally well. It is, therefore, apparent that strains of the species *Salmonella pullorum* may show as much variation in agglutinability as those of *Eberthella typhi*.

It is well to call attention to the fact that the environmental conditions under which bacteria are grown and antigens are produced play an important part in their agglutinability. A strain may vary considerably in its agglutinability when grown on media of different hydrogen ion concentration or sugar content (Ishii (1922a)). The kind and concentration of salts in the suspending menstruum play an extremely important rôle (Northrop and DeKruif (1922)). Therefore, before one can conclude that variations exist between strains of a species it must be shown that all environmental conditions are uniform. It seems that the variations reported, both for *Eberthella typhi* and *Salmonella pullorum* occurred under uniform environmental conditions. It is true that each group of investigators used a different combination of environmental conditions but consideration must be given to the fact that each supposedly maintained uniformity throughout his investigation.

Bordet's (1899) observation that bacteria are not agglutinated by immune serum unless salts are present placed the phenomenon of bacterial agglutination upon a colloidal basis. He showed that specific bacterial agglutination consists of two phases, first, the specific adsorption of agglutinin by the bacteria, and, second, the agglutination or precipitation of the new complex by the salt. Agglutinin, therefore, itself, does not cause agglutination, but when it is specifically adsorbed by the bacterial cells it forms an unstable complex which is precipitated by the action of the electrolyte of the suspending menstruum. This change of behavior resembles the change from a stable hydrophilic colloid

to a non-stable hydrophobic colloid which is sensitive to electrolytes.

Many investigators have found that colloids, when suspended in water, are electrically charged. It has also been shown that when placed in an electric field they migrate generally toward the anode, that is, that they are negatively charged. The stability of colloidal suspensions is dependent upon two forces, namely, repulsion and attraction. The mutual repulsion of the cells is due to like charges. On the other hand, surface tension which strives to reduce the free surface by bringing the particles together acts as the attractive force. Both forces are constantly at work, except at an absolute isoelectric point. So long as the repelling force is the greater, the particles remain in suspension but if the electric charge is reduced to a point or zone where the attractive force becomes the greater, precipitation or agglutination occurs.

It has been shown in the summaries of Winslow, Falk and Caulfield (1923) and Winslow and Upton (1926) that the relative charge carried by bacteria can be measured by the relative electrophoretic migration and that suspensions of bacteria as ordinarily grown are negatively charged. Bacteria remain in suspension because of the repelling action of the like electric charges which they carry, an action which opposes the attractive force, or, as Northrop and DeKruif (1922) prefer to say, the cohesive force. These investigators measured both the electrophoretic potential and the cohesive force and found that whenever the potential difference between the surface of the bacterium and the suspending menstruum was reduced to about 15 millivolts the bacteria agglutinated, provided that the cohesive force was not affected. If the cohesive force was reduced, the critical potential had to be lowered and if the cohesive force was made very small no agglutination occurred even if the potential was reduced to zero. They found that bacteria sensitized with immune serum had a constant cohesive force and, therefore, it seemed to us that any difference in the relative ability of strains to be agglutinated must be due to differences in the relative electrophoretic potential, practically to differences in relative electrophoretic velocity.

During the progress of our work Falk, Jacobson and Gussin (1925 a, b and c; 1926 a and b) reported upon a study of the relation between electrophoretic migration velocity, virulence, lethality and agglutinability of the groups of pneumococci. Their results suggest that electrophoretic potential is related in some fundamental manner to agglutinability since they found a sequence of decreasing agglutinability accompanied by a sequence of increasing electrophoretic potential.

OBJECTIVES AND GENERAL RESULTS OF THE PRESENT INVESTIGATION

The investigation reported here was begun during the summer of 1925 to determine, first, if strains of *Salmonella pullorum* vary with respect to their electrophoretic migration velocity, and, second, in case variations were found, to study the quantitative relationship to agglutinability.

Our results show that the species *Salmonella pullorum* bears a negative charge and it is clearly evident that great variations exist between strains so far as quantity of charge is concerned as evidenced by the differences in the rate of electrophoretic migration velocity. Variations were also found in the agglutinating ability of strains. In general, the sequence of decreasing electrophoretic migration velocity was identical with the sequence of increasing agglutinating ability, therefore, there appears to be a quantitative relation between the electrophoretic velocity and the agglutinating ability of strains of this species. This, we believe, explains at least in part, why some strains of a bacterial species are more easily agglutinated than others.

TECHNIQUE

Preparation of the suspension. Proteose-peptone agar adjusted to approximately pH 7.0 has been successfully and conveniently used for the cultivation of *Salmonella pullorum* by the senior author during the past several years and, therefore, was considered suitable for this investigation. In preparing this medium Difco proteose-peptone is substituted for ordinary pep-

tone in nutrient agar. Since Shibley (1924) found that the charge carried by *Esch. coli* varied until the culture was about eighteen hours old, it was deemed advisable to use a constant incubation period of twenty-four hours. The bacteria were grown upon slants at 37°C. The growth was removed with a small volume of 0.85 per cent NaCl by a gentle rotary movement of the culture tube. The suspension was then shaken with glass beads to break up clumps of bacteria. This suspension was filtered through cotton to remove flakes of agar, centrifuged at about 2500 revolutions per minute and the cells resuspended in 0.87 per cent NaCl. The washing was repeated three times to remove traces of peptone, etc., because DeKruif (1921) found that such impurities affected both the quantity of charge and the agglutinating ability. The final washed suspension was heated for one hour at 56° to 58°C. and standardized to a density which corresponded to approximately 3 cm. by the Gates (1920) method. The suspension thus prepared was used for electrophoretic measurements and agglutination tests, both being made the same day that the suspension was prepared.

Measurements of the electrophoretic migration. The apparatus used in this investigation was similar to that devised by Northrop (1922) and of the type now being prepared by the Eimer and Amend Company of New York.

We found that the use of a trace of dye (methylene blue) in the zinc sulphate solution greatly facilitated the filling of the electrode chambers and prevented an excess of zinc sulphate being run into the electrophoresis cell as the colored solution could be seen within the stop-cocks with much greater ease than the colorless one. Before each sample was introduced into the apparatus, the cell was washed with at least one liter of distilled water in order to remove even the slightest amount of zinc sulphate which might have entered when the electrode chambers were filled. During preliminary experiments we found that unless the distilled water was removed completely with NaCl solution of the same concentration as that in which the bacteria were suspended, diffusion currents were produced which prevented the making of readings. Therefore, after the cell was

washed with distilled water it was flushed with about 200 cc. of 0.85 per cent NaCl and finally with about 50 cc. of the bacterial suspension.

A direct current of approximately 60 volts was applied, which gave a potential gradient of approximately 2.1 volts per centimeter. Observations were made with direct illumination and a magnification of 525 diameters. All measurements of electrophoretic velocity were made by timing with a stop-watch the migration of a given bacterium across a distance of 32 micra.

Preliminary readings, made in order to determine the depth of the cell and the level of maximum velocity taken at each one-sixteenth of the depth of the cell, when plotted, reproduced the Smoluchowski (1921) curve. This suggested that four readings be made between six-sixteenths and nine-sixteenths of the depth of the cell, each differing by one-sixteenth, because at these levels the migration was fastest. The advantage to be gained by making these four readings, rather than readings at a single level or at certain specific levels as others have done, lies in the fact that a greater confidence is had in the accuracy of the values obtained, for they can be plotted and it can be observed whether or not the curve obtained passes through a maximum. All velocities reported in this paper actually did this very thing. Readings were made upon 20 cells at each level and an average of the 80 readings are reported. The current was reversed after the fifth, tenth and fifteenth readings at each level. We believe that this system of reversing the current is of distinct advantage because even the slightest amount of drifting can be detected easily by unequal migrations in the two directions. The results of our investigation are expressed in terms of micra per second per volt per centimeter since the Helmholtz-Lamb equation used to convert electrophoretic migration velocity into potential difference is questionable as discussed by Winslow, Falk and Caulfield (1923).

Determination of the agglutinating ability. Antisera from two rabbits immunized with strain X-43 were used. The usual procedure for macroscopic agglutination tests was employed. The NaCl needed for making serum dilutions was taken from

the same lot as that used to prepare the bacterial suspensions. The serum was diluted to each dilution in large tubes and then pipetted out to the agglutination tubes so that all suspensions would be mixed with serum of exactly the same strength. Readings were made after forty-eight hours incubation at 37°C. The results are presented according to the method advocated by Hadley (1917).

Cultures. The 18 strains used were from widely separated sources and dates of isolation. Half of the strains were isolated by the senior author. A-40 and X-61 were from infected ovaries. X-48 was from the heart of a hen. X-57 was from the heart of a three-month-old chick. All other strains were from either the liver or heart of chicks. All strains were transferred daily for three days before they were used in this work.

EXPERIMENTAL RESULTS

Our first experiments, made to determine the experimental error, showed that almost identical average migration velocities could be obtained when several suspensions were prepared from a strain, as indicated in table 1. These suspensions were prepared as described above but from separate lots of agar slants. An inspection of the data shows that the average migration velocity of any suspension did not vary more than approximately 3 per cent from the average of all suspensions of that strain, thus confirming the accuracy of the method as reported by Falk and Reed (1926).

The results of both electrophoretic measurements and agglutination tests made upon 18 strains are summarized in table 2.

The strains were grouped according to their agglutinabilities. In general, the greatest dilution of serum at which complete agglutination still occurred (titre) was: for group one, about 100; for group two, 800; for group three, 1600; for group four, 3200 and for the homologous strain X-43, 3200. The average electrophoretic migration velocities were: for group one, -1.578; for group two, -1.069; for group three, -0.955; for group four, -0.722 and for the homologous strain, -1.157. This indicates

TABLE 1

Average migration velocities (in micra per volt per centimeter) of repeated experiments

STRAIN	SAMPLE	VELOCITY	AVERAGE OF ALL STRAINS	VARIATIONS FROM THE AVERAGE
X-40	a	−1.123		0.028
X-40	b	−1.068	−1.095	0.027
X-43	a	−1.058		0.008
X-43	b	−1.078		0.028
X-43	c	−1.015	−1.050	0.035

TABLE 2

Migration velocities and agglutination tests

STRAIN	MIGRATION VELOCITY*	AVERAGE OF GROUPS	AGGLUTINATION									SOURCE OF CULTURE
			Serum dilutions									
			1	2	3	4	5	6	7	8	9†	
X-48	−1.714		3	1	0	0	0	0	0	0	0	Heart of hen
B-93	−1.280		4	3	1	0	0	0	0	0	0	Chick
X-57	−1.740	−1.578	4	4	4	2	1	0	0	0	0	Heart of three-month-old chick
A-40	−1.176		4	4	4	4	3	2	1	0	0	Ovary
A-33	−1.138		4	4	4	4	3	2	0	0	0	Chick
X-61	−1.100		4	4	4	4	3	1	0	0	0	Ovary
B-64	−1.019		4	4	4	4	2	1	0	0	0	Heart of hen
A-24	−0.985		c	c	4	4	3	2	1	0	0	Chick
B-104	−0.995	−1.069	4	4	4	4	3	2	1	0	0	Chick
A-12	−0.995		c	c	c	4	4	2	2	0	0	Chick
X-80	−0.964		4	4	4	4	4	2	1	0	0	Chick
B-71	−1.047		4	4	4	4	4	3	1	0	0	Chick
X-49	−0.955	−0.955	4	4	4	4	4	3	1	0	0	Chick
X-52	−0.993		4	4	c	c	4	4	3	0	0	Chick
B-73	−0.646		c	c	4	4	4	4	3	1	0	Chick
B-74	−0.627	−0.722	c	c	c	c	4	4	3	2	0	Chick
X-43‡	−1.157	−1.157	c	c	c	c	4	4	2	1	0	Chick

* Each average migration velocity represents the average of 80 stop-watch readings.

† Serum dilutions 1:100, 1:200, 1:400, 1:800, etc., represented by 1, 2, 3, 4, etc.

‡ Homologous strain.

very clearly a relationship between the electrophoretic migration velocity and the agglutinability of the species *Sal. pullorum*, since there is a sequence of increasing agglutinability which is accompanied by a sequence of decreasing migration velocity. The apparently abnormal behavior of the homologous strain X-43 will be discussed later.

It is interesting to note that strains isolated from mature birds showed relatively poor agglutinability and a high charge.

DISCUSSION

Since electrophoretic migration velocities can be measured to an accuracy of 3 per cent and since a total variation of approximately 63 per cent was obtained, it appears that the variations in electrophoretic results reported are really significant in view of the fact that these variations between strains were observed under uniform environmental conditions, the medium, the 0.85 per cent NaCl, the procedure of making both suspensions and electrophoretic measurements, the pH and the temperature being practically identical in every case.

That the experimental results should reveal relationship between agglutinability and electrophoretic migration velocity was expected as a result of an application of fundamental colloid chemistry. Since bacteria behave as hydrophilic colloids which are changed into hydrophobic colloids by immune serum (Wells (1925)) and since the agglutination of the hydrophobic particles is a question of bringing them into an iso-electric zone (Northrop and DeKruif (1922)), it follows that the charge which is represented by electrophoretic migration velocities must vary in some way with agglutination tendencies. It is, therefore, not surprising, when all experimental conditions except the nature of the organism are kept constant, to find that the series representing the agglutinability and that representing the electrophoretic migration velocities appear to be comparable.

Strain X-43 did not lend itself to a proper placement in the series. In view of the work of Shibley (1924) and the statement made by Falk, Jacobson and Gussin (1926a) namely that "the type specificity of each serum is as plainly evident in the reduc-

tion of electrophoretic potential as in the agglutination of the bacteria," it appears that we should not have expected a placement which would have put agglutinability and electrophoretic migration velocity in the same relative position. The placement that was obtained is just that which is to be expected from the above work, namely, better agglutinability than the charge suggests.

That the readings obtained with strains isolated from mature birds showed relatively poor agglutinability and high charge is interesting in view of the work published by Falk, Jacobson and Gussin (1925a) in which they called attention to the fact that, for the pneumococci, charge and virulence paralleled each other. Such a parallelism is suggested here on the assumption that strains from the adults possess more virulence than those from chicks.

Attention is called to the fact that work is now in progress in which an attempt is being made to vary the charge of the organism by varying the components of the medium upon which it is grown, in order to ascertain whether variations in agglutinability and charge can be given to the organism by the constituents of the growth medium to such a degree that they can be carried over when the organism is suspended in various suspending menstra. This work should throw some light on why different strains should have different agglutinabilities and different charge, and on whether work in this field can be duplicated provided workers do not use exactly the same growth and suspending media.

SUMMARY

1. The use of a dye in the solution used to fill the electrode chambers facilitated the filling of the electrophoresis apparatus.

2. The Smoluchowski curve was obtained.

3. It is suggested that greater confidence in readings is obtained by making measurements which will span the hump of the above curve.

4. Electrophoretic migration velocities were determined with an accuracy of 3 per cent.

5. Eighteen strains of *Sal. pullorum* studied carried a negative charge.

6. Variations of over 60 per cent in the electrophoretic migration velocities and a titre ranging from about 100 to 3200 were obtained.

7. In general, the sequence of decreasing electrophoretic migration velocity followed the sequence of increasing agglutinability.

8. The use of homologous serum produced an agglutinability superior to that suggested by the charge.

9. A relationship between electrophoresis, agglutinability and virulence is suggested.

10. Attention is called to work in progress.

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MICROBIAL THERMOGENESIS

II. HEAT PRODUCTION IN MOIST ORGANIC MATERIALS WITH SPECIAL REFERENCE TO THE PART PLAYED BY MICROÖRGANISMS[1]

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INTRODUCTION

The growth of microörganisms upon certain types of substrates has been shown to be accompanied by the liberation of measurable quantities of heat. Rubner (1904) and Mohr (1914) studying fermentation by different strains of yeast calculated the number of calories of heat liberated per gram of sugar fermented. Shearer (1921), growing *Bact. coli* in tryptic broth, peptone and glucose solutions, recorded the number of calories of heat produced; he obtained the greatest amount in a peptone-glucose-chalk solution. He found that very little heat is liberated in an amino-acid substrate; yet only about 1 per cent of the total energy available in the carbohydrate molecule is used. Other studies which are equally interesting have shown that in the curdling of milk the greatest amount of heat is liberated after the period of acid formation (Rubner (1907)), and also that appreciable calories are liberated in antigen-antibody reactions. Bayne-Jones (1925) measured the heat produced by the union of diphtheria toxin and antitoxin and by the agglutination of dead bacilli.

Studies of the above nature have been conducted in liquid media possessing a comparatively high specific heat, the 2° or 3° observed rise in temperature failing to affect the rate or type of microbic development. Should this amount of heat be liberated

[1] This investigation was begun in the Bureau of Chemistry, Washington, D. C., and was continued and completed in the Laboratory of General Bacteriology, Yale University, where it was aided by a Sterling Fellowship grant.

in an insulated chamber containing a medium which absorbs but a small percentage of the heat produced, the rise in temperature might become far more significant. Cohn (1888) was the first worker to study the thermogenic powers of microörganisms, also the first to apply the term "thermogenesis." In mixed cultures Cohn attributes the heat production below 35°C. to plant cell respiration and above 35° to molds. In 1893 he reported observing a temperature of 67.2°C. in raw cotton.

To our knowledge, but one investigator has attempted to study the thermogenic powers of pure cultures of microörganisms. Miehe (1907) devised a graduated series of wire chambers, the innermost to contain the test material and others to prevent contamination and to furnish insulation. Chopped hay packed into the smallest container and "sterilized" in steam for one hour was inoculated with a liquid suspension of the test organism, packed into the other cylinders and temperature changes observed upon a thermometer. *B. calefactor*, which Miehe isolated from heating hay, raised the temperature of sterilized material from 40°C. (incubator temperature) to 46°C. He concludes that since *B. calefactor* will grow at temperatures as high as 75°C., it may be responsible for the heating of hay stacks to that temperature. Miehe also reports that *Oidium lactis* produced a temperature of 58.5° and *Asper. niger* of 48.0°C.

The ability of microörganisms to produce comparatively high temperatures has been assumed by various investigators—Holdefleiss (1899), Dügelli (1906), Suchsland (1894), Behrens (1894, 1897 and 1901), Laupper (1920), Hunter (1917) and others attributing the heating of stored organic materials in whole or in part to the activities of microscopic organisms. Certain workers, Burri (1919), Tschirch (1917), and others referred to elsewhere, admit microörganisms to no part in the heating of stored moist materials. Haldane and Makgill (1923), accepting Miehe's explanation of the heating to 70°C., analyzed the gases given off by hay aerated in a flask held in a constant temperature bath and concluded that a simple chemical oxidation occurred, besides microbial activity.

The phenomenon of "spontaneous" heating of farm products

has been observed for decades past; the heat produced in stable manure and moist hay in particular was reported years ago. The utilization of the heat liberated from fresh stable manure for the growing of mushrooms, and in hot-beds, the heating of stacks of bark in the manufacture of white lead, and the seasoning of tobacco, tea and coffee during the normal sweat of the plant products, are industrial applications of the phenomenon commonly known as "spontaneous heat production." Not all instances of "spontaneous" heating are desirable; the scorching of hays, grains, fodders, etc., is distinctly detrimental to the market values of the products. There are many types of heating which are quite desirable so long as they occur in moderate degree. However, if allowed to proceed beyond a recognized limit, they may become quite harmful.

That fires result from such heating can no longer be doubted. Laupper (1923) reported nine cases in Germany, Heaton (1921) sixteen prior to 1921, and twenty-two during that year in Canada, and Cottrell (1902) six cases as representative of "many instances during the summer of 1901" in the State of Kansas. Conclusive detailed reports of single instances of "spontaneous" combustion have been furnished by Ranke (1873), Schwarz (1921) Laupper (1924) and others. Heating is generally recognized as appearing in several stages; the first or initial heat occurs rather rapidly soon after the material is massed together and attains a temperature of usually not over 80°C. Since practically all organic materials[2] stored under normal conditions harbor bacteria and molds, the determination of the heat-producing powers of these microörganisms is a logical starting point for investigation.

EXPERIMENTAL

Raw products

Various types of apparatus for the quantitative determination of the calories of heat produced per unit of sugar fermentation

[2] "Organic materials" is used throughout this manuscript to include those plant or animal substances which when piled produce a porous mass and excludes the watery substances not naturally falling within the purpose of the investigation.

have been described by Rubner (1903), Hill (1911), Körösy (1913) and others. The apparatus used in our studies (James,

TABLE 1

The effect of different percentages of moisture upon the rate of heat production and the maximum temperature produced in cornmeal

All flasks were aerated

DAYS	HOUR	ROOM TEMPERATURE	TEMPERATURE								
			Dewar, No. 1, 12 per cent	Dewar, No. 2, 15 per cent	Dewar, No. 3, 18 per cent	Dewar, No. 4, 21 per cent	Dewar, No. 5, 24 per cent	Dewar, No. 6, 27 per cent	Dewar, No. 7, 30 per cent	Dewar, No. 8, 35 per cent	Dewar, No. 9, 40 per cent
1924											
0	4:30 p.m.	27.0	21.3	23.4	24.5	24.3	24.2	23.9	28.0	28.2	25.4
1	10:00 a.m.	27.2	25.4	26.2	26.9	26.4	26.7	27.1	29.4	29.5	27.5
	4:30 p.m.	30.0	26.3	26.9	27.4	26.8	27.2	27.9	30.9	31.2	30.8
2	9:00 a.m.	27.0	27.8	28.0	28.0	27.4	29.4	36.5	47.0	46.4	35.4
	4:30 p.m.	28.0	27.7	27.8	27.6	27.4	33.6	44.0	49.7	47.0	40.5
3	9:00 a.m.	26.5	27.2	27.2	26.9	28.6	44.6	44.9	54.2	53.6	37.8
	5:00 a.m.	27.0	27.2	27.2	26.9	30.9	44.6	46.6			
4	9:00 a.m.	25.0	27.0	26.8	26.9	36.3	43.4	45.0	54.8	53.2	30.2
	4:00 p.m.	25.0	26.3	26.0	26.4	38.5	42.3	48.2	54.9	53.5	
5	9:00 a.m.	25.0	25.4	25.3	27.6	43.1	50.5	54.6	57.6	54.5	29.0
	4:30 p.m.	25.5	25.3	25.2	29.0	43.4	51.5	56.1	58.4	54.4	
6	10:00 a.m.	24.0	24.9	24.9	33.4	44.6	53.2	56.7	61.8	56.2	30.7
7	9:00 a.m.	22.5	23.2	23.1	36.1	48.2	55.7	58.8	61.4	56.9	35.3
	5:00 p.m.	23.5	23.2	23.2	37.3	49.4	56.9	58.2	62.1	58.4	36.7
8	9:00 a.m.	23.0	23.3	23.4	37.0	50.0	58.7	60.8		60.9	37.0
	9:00 p.m.	26.0			37.3	50.9	61.1	62.4		60.7	
9	9:00 a.m.	25.5			38.6	50.7	56.6	61.9		60.7	37.4
	4:00 p.m.	25.5			39.0	50.9	58.8	60.9			
10	9:00 a.m.	25.2			38.7	52.5	61.4	46.5			40.4
11	9:00 a.m.	25.0			38.7	56.0					

TABLE 1—*Continued*

DAYS	HOUR	ROOM TEMPERATURE	TEMPERATURE								
			Dewar, No. 1, 12 per cent	Dewar, No. 2, 15 per cent	Dewar, No. 3, 18 per cent	Dewar, No. 4, 21 per cent	Dewar, No. 5, 24 per cent	Dewar, No. 6, 27 per cent	Dewar, No. 7, 30 per cent	Dewar, No. 8, 35 per cent	Dewar, No. 9, 40 per cent
1924											
12	9:00 a.m.	26.0				59.5					43.0
	3:00 p.m.	26.0				60.7					42.4
13	12:00	25.5				62.0					43.0
14	9:00 a.m.	25.0				61.7					45.4
24	9:00 a.m.	25.0									50.1
33	9:00 a.m.	25.5									52.0

1927) furnished means of aerating the test material with any desired gas. Oxygen, used throughout the course of this work, was forced into the mass of the test substance from a large bottle by the slow introduction of water, the rate of flow being governed by a capillary valve.

Moistened cornmeal and cracked field corn heated readily when aerated in the flasks in this apparatus. Many experiments have confirmed the observations noted in the previous report (James 1927), namely, that heating does not take place in the absence of air or oxygen. Since the rate of microbic growth and intracellular enzymic activity, as well as the specific heat of the material, would be materially affected by the amount of water present, the moisture content optimum for the heating of commercial cornmeal was determined. Nine flasks were packed with equal quantities of meal that were similarly treated except that the amount of water varied. The results of the experiment (table 1) indicate that 30 per cent is the optimum moisture content, the maximum temperature 62.+°C., being attained in the shortest time. The meal in the flasks which had shown no heating was unchanged in its macroscopic appearance, while in all flasks where a rise in temperature had been recorded mold development was observed.

In order to confirm the supposition that microbic development does occur during the heating, sixteen flasks were packed with

TABLE 2

The microbial condition and acidity of unsterilized cornmeal at various stages of the rise in temperature

FLASK NUMBER	TIME OF INCUBATION	MAXIMUM TEMPERATURE		BACTERIAL COUNT PER GRAM OF MEAL		MOLD COLONIES APPEARING ON WORT PLATES PER GRAM OF MEAL	pH
				30°C.	50°C.		
	days	°C.					
Fresh meal	0			15,000			
1	1	Room*		20,000			6.0
2	1	Room		500,000			6.0
3	2	28.1		1,100,000			6.0
4	2	31.2		40,000,000			6.0
5	3	42.3		1,000,000			5.4
6	3	43.5		60,000,000			5.4
7	4	51.3		1,100,000		30,000	5.2
8	4	51.5		450,000		20,000	5.2
9	5	48.0		16,000,000		200,000	5.0
10	5	51.8		100		20,000	5.0
11	8	55.8	A†	0	22,000	14,000	5.0
			B	0	85,000	15,000	
12	8	55.6	A	0	23,000	8,000	
			B	0	400,000	40,000	5.4
13	12	62.0‡	A	0	0	0	
			B	3,000	0	0	4.4
14	7	61.1	A	600	250	260	
			B	1,100	2,600	900	4.8
15	24	62.3§	A	0	500	500	
			B	0	6,000	500,000	4.2
16	7	61.4	A	0	10	80	
			B	10,000	6,800	170	5.0

* Figures indicate the temperature attained by the flask at the time of examination.

† "A" = sample from center of flask, in less discolored area. "B" = sample adjoining wall of flask.

‡ Approximately this temperature maintained for four days.

§ Approximately this temperature maintained for fourteen days.

moist cornmeal and aerated in the apparatus. At selected periods during the rise in temperature the contents of two flasks

were examined bacteriologically. Five-gram subsamples were thoroughly washed in sterile water (sterile sand was added when necessary) and aliquot portions (diluted and undiluted) were seeded into wort agar and duplicate glucose agar plates. The wort medium was incubated at 30°C. and the sugar agar at 30° and 50°C. The H ion concentration of the meal was determined as accurately as the use of several indicators permitted.

The data collected from these experiments and listed in table 2 bring out several pertinent facts. The organisms growing at 30°C. increased in numbers in the meal until the temperature within the flask had reached about 50°C. When the temperature attained 55° or above, markedly lower counts were obtained. Incubation of glucose agar plates at 50°C. was not begun until the temperature had reached 55°C. Mold counts on wort agar plates, begun with meal at a temperature of about 50°, show a consistent decrease except in flask 15, which contained a considerable growth of a thermophilic mold (*Asper. fumigatus*). Subsamples of meal removed from the center and the wall of flasks 11 to 16, inclusive, were plated separately. In every instance the count of the meal adjacent to the wall was higher, though temperatures in this area were usually found to be the same as in the center of the flask. Where differences of temperature did appear, that in the center was slightly lower.

Mold growth coincident with bacterial development in unsterilized materials may remove acid liberated as a byproduct of bacterial metabolism. Thus, bacterial growth may be prolonged and the temperature maintained at a maximum for a considerable period of time. In order to determine the duration of a high temperature in a mixed culture, Dewar 13 (table 2) was aerated for four days and Dewar 15 for fourteen days, after the maximum of 62.+° was reached. The former was still at 62.+ when examined, while the latter, after maintaining a maximum of 62.+° for fourteen days, had decreased to a temperature of 52.0°C. The fall in temperature is assumed to be due to a depletion of available food materials. That all of the acid was not removed by the mold is shown by the increase in acidity with longer incubation periods. Dewar 15 had a final pH

of 4.2 as compared with a pH of approximately 6.0 in the corn before heating.

Threshed wheat and oats are susceptible to considerable heating and deterioration when stored in a moist condition. They heat less readily, however, than cracked corn, etc., in which abundant food material is easily accessible to microörganisms. In a series of reports Bailey (1917a, 1917b and 1921) and his co-workers (Bailey and Gurjar (1918 and 1920)) point out the relation between heating and the moisture content of grains, holding that microörganisms play no part in the phenomenon. When packed into the apparatus, moistened winter wheat heated to 40.6°C. when aerated with oxygen, and to 33.7° when not aerated, while moistened oats heated to 48.3° and 49.2° when aerated and to only 39.1° when unaerated. The greater amount of heating evidenced in unaerated wheat and oats than in unaerated cornmeal (32.7°C.) is probably induced by the greater amount of air entrapped during the packing. It is significant that with cornmeal the maximum temperature is usually obtained within seven to nine days after packing, while with wheat and oats the highest temperatures were not observed until after twelve and thirteen days incubation, respectively.

The "spontaneous" heating of moist or uncured hay is more dangerous than that of any other organic material. The actual cause of fires is not known, though the rapidity with which some highly inflammable substance is produced is considered significant. Cured meadow hay when moistened and aerated in the apparatus heated as follows:

Hay of 40 per cent moisture heated to 55.2°C.
Hay of 56 per cent moisture heated to 60.0°C.
Hay of 61 per cent moisture heated to 57.4°C.

No highly inflammable material was observed, due possibly to the fact that none of the experiments lasted over seven days.

Extensive studies on the deterioration of field corn during transit or storage have been made by the Grain Standardization Laboratory of the United States Department of Agriculture. Duvel (1909), and Duvel and Duval (1911 and 1913) correlated

the degree and rapidity of spoilage with the moisture content of the corn. Boerner (1919) made observations upon the factors influencing the carrying qualities of corn when exported to foreign countries. Nine cargoes were accompanied to their respective European destinations, and daily temperature observations showed again that the amount of heating and spoilage was closely related to the initial moisture content of the corn.

The results of many experiments with cracked corn in our apparatus closely resembled those previously reported (James (1927)) and demonstrated the readiness with which this product will heat. It was of interest to determine the moisture content

TABLE 3

Highest temperature produced in unsterilized commercial cracked corn containing various percentages of moisture

MOISTURE CONTENT	MAXIMUM TEMPERATURE	INCUBATION	MOISTURE CONTENT	MAXIMUM TEMPERATURE	INCUBATION
per cent	*°C.*	*days*	*per cent*	*°C.*	*days*
16	42.4	9	32	62.5	5
21	61.7	11	32	46.3	3
24	54.3	2	34	62.6	9
27	54.3	3	34	41.7	3
27	56.5	3	39	50.0	4
27	57.3	3	39	46.6	5
27	62.6	6	39	53.2	4
28	61.3	16	42	55.9	2
30	46.0	6			

optimum for the "spontaneous" heat production under the conditions provided in the apparatus. Seventeen flasks of corn, packed and held in the apparatus, yielded the results listed in table 3. The highest temperatures obtained were 62.+°C. While the maximum temperatures produced appear to be only slightly affected by the amount of water present, it is obvious that high temperatures may be produced over a wide range of water content. Though accurate control of the moisture content was not necessary, in order that the results may be fully comparable 30 per cent was selected as the most favorable for further experimentation. The wide differences between the maximum temperatures recorded in lots of corn of the same mois-

ture content and similarly treated indicate that the height of the temperature rise is dependent, not upon the activity of the plant enzymes, but upon microörganic growth. Different species of organisms probably predominate in different portions of the corn.

The amount of material which can be packed into a one-quart Dewar flask being comparatively small, the possibility arises that the heat losses through radiation and aeration may form a considerable part of the total heat produced. In order to determine whether or not oxygen aeration of larger insulated masses will produce higher maximum temperatures, test materials were packed into 20-gallon earthen crocks and aerated. The crocks were insulated in several inches of sawdust and provided with closely fitting wooden covers which were lined with four layers of heavy asbestos board. Oxygen was introduced from a pressure tank by means of a copper tube which opened in the center near the bottom of the mass. A water bubbler was interposed to indicate roughly the rate of gas flow.

Moistened, ground hay, when packed into the crocks, heated only when supplied with oxygen. The results of two tests, one without introduced oxygen, show that the maximum temperature obtained by aeration (61.2°) is approximately the same as that recorded with smaller masses of material. This maximum was observed at the bottom (near the oxygen inlet) of the mass. The lower layers of the unaerated material did not heat to 30.0°C., while the upper layers, which were exposed to the air during the periodic temperature observations, heated to 43.0°C. The conclusion is inevitable that oxygen is necessary for "spontaneous" heat production. This is further substantiated by the sudden fall in the temperature of the aerated mass when the oxygen was shut off.

While the moistening of cured materials may restore the original water content, it probably does not reestablish the plant enzymes to their original state of activity. If such were the case the packing of uncured materials into the apparatus should lead either to the production of a higher temperature or to the attainment of the maximum temperature in a shorter period of time than is the case with moistened cured substances. Uncured

alfalfa, when packed and aerated in the large crocks, heated to 60.+°C. in eight days, in approximately the same time and at about the same temperature as noted with moistened cured hay. Thus the agents active in the heat production probably are not the plant tissue enzymes, but microörganisms growing upon the available food substances.

The decrease in the value of stable manure for fertilizer purposes when allowed to heat has lead to the adoption of methods to preserve its nitrogen content, i.e., to prevent heating. When fresh stable manure was packed into two of the crocks and not aerated with oxygen, the results resembled those obtained with hay, i.e., the lower layers of each mass remained below 30.0°, while the upper layers heated to 48.0° and 49.5°C., respectively. When aerated with oxygen, maxima of 71.0° and 73.5° were obtained, the highest temperature recorded for any material used in these studies.

PURE CULTURES

Whether or not microörganisms are significant in so-called "spontaneous" heat production has long been a moot question. Data presented above strongly indicate that they play a very important rôle, at least in the early stages of the heating. In the above experiments intracellular activity of the plant cells undoubtedly played some part. Microörganisms growing in pure culture under conditions in which the major portion of heat produced is retained may be made to demonstrate their powers of thermogenesis.

Miehe (1907) attempted such a demonstration by inoculating "sterilized" hay with suspensions of microörganisms, as stated above. While the conditions of his experiments more nearly approximate absolute control of the factors influencing heat production than do those of any other investigator, yet even his work can not be claimed to have proven the ability of pure cultures to produce appreciable rises in the temperature of the substrate.

In selecting a medium upon which to grow the organisms several factors had to be considered. The medium should be,

(1) as low in specific heat as possible; (2) as porous as possible in order to allow ready diffusion of the introduced oxygen, and to be readily sterilizable; (3) high in available carbohydrate, since the heat has been shown to come largely from that component; and (4) a material known to be suitable for microbial growth. Cracked field corn more nearly met these requirements than any other material tested.

Thorough sterilization of the cracked corn in flasks in a comparatively dry state was found to require over one hour's heating in the autoclave at 15 pounds extra pressure. Corn so treated is usually badly scorched and unsuited to our purposes. The following technic of sterilization was therefore adopted and found to be fully reliable. A thin layer of about 250 grams of commercial cracked corn is heated in a shallow pan in an oven at 150°C. for twenty minutes. Having attained the temperature of the oven during this exposure the corn is transferred immediately to a wire cylinder made of fine copper mesh and shaped to fit inside the metal case of a commercial 1-quart Dewar flask. The mouth of this case closely corresponds to that of a three-liter Pyrex Erlenmeyer flask. The hot corn in the wire holder and the metal case[3] are heated in the autoclave at 15 pounds extra pressure for twenty minutes. The corn is then poured immediately into a sterile three-liter Erlenmeyer flask.

During sterilization the glass containers and the metal case are cotton-plugged and, together with the wire cylinder containing the corn, are covered by inverted paper bags. Corn so treated, only slightly scorched, is left to cool in the stoppered flask. Since the test organism is inoculated onto the sterilized corn in the form of a water suspension, a glass tube passing through the cotton stopper of the Erlenmeyer flask permits seeding the corn without removal of the stopper.

As previously noted (James, 1927), sterilization of the ordinary 1-quart vacuum bottle is impractical, and a hard glass cylinder fitting snugly inside a wide-mouthed "food-jar" type of Dewar flask is recommended to hold the inoculated material. The

[3] During sterilization the wire cylinder containing the corn is placed *beside* the metal case and inserted after the autoclave is opened.

mouth of this cylinder having been drawn to conform to that of a three-liter Erlenmeyer flask, the inoculated corn is transferred to the sterile cylinder with little chance of contamination, precautions of flaming the mouth of each being observed. That corn can be handled without contamination by the above technic has been proven many times by the subsequent addition of sterile glucose broth and its failure on incubation to show the growth of any microörganisms.

In determining the thermogenic powers of single strains of microörganisms, the purity of the inoculum and the purity and identity of the organisms reisolated from the corn after the heating has taken place have always been carefully established. Such check measures are essential, since the chance entrance of a single mold or bacterial spore may lead to the organism's permeation through the entire medium.

In order to obtain organisms capable of growing upon the corn kernel, strains were isolated from those predominating in heating unsterilized cornmeal and cracked corn. In the early part of the experimental work isolations were made and maintained on glucose agar. The inoculum for seeding the corn was washed with sterile water from glucose agar slants, several slants being washed with the same water in order to obtain a very heavy suspension. Sufficient of this suspension was then added to the sterile corn to bring its moisture content to between 30 and 35 per cent. This was then packed into the apparatus in the hard glass cylinder.

Before presenting data of tests with microörganisms it is necessary to show that the same set-up, lacking only the living cells, will not heat. When sterile cracked corn moistened with sterile water is packed into the apparatus and held, with or without oxygen aeration, no heating whatsoever takes place.

The thermogenic powers of twelve strains of organisms isolated largely from cornmeal have been determined. It should be noted that the heating powers of a single strain may vary under apparently similar conditions, and certainly under different conditions of experimentation. The maximum temperatures recorded in table 4 are the highest observed for each strain under

the respective conditions. Lower maxima under apparently similar conditions have been noted in other experiments and will be referred to later.

Reference to table 4 shows primarily that pure cultures of some bacteria are powerfully thermogenic under certain conditions. Six strains have been isolated which were capable of raising the

TABLE 4

Highest temperature produced by pure cultures under different conditions of experimentation

Corn in all tests contained about 30 per cent moisture and was aerated with oxygen

ORGANISM	IDENTITY	TEMPERATURE OF ISOLATION	INOCULUM GROWTH TEMPERATURE	TEMPERATURE OUTSIDE FLASK	MAXIMUM TEMPERATURE PRODUCED
		°C.	°C.		
1	*B. subtilis*	30	30	Room	53.9
1	*B. subtilis*	30	37	Room	48.9
2	———*	30	30	Room	47.1
3	*Proteus vulgaris*	50	30	Room	54.1
3	*Proteus vulgaris*		30	Room	32.2
3	*Proteus vulgaris*		37	Room	36.4
4	*B. vulgatus*	50	30	Room	26.2
5	(Mold)*	30	30	Room	46.5
6	*B. vulgatus*	50	30	Room	57.5
6	*B. vulgatus*	50	37	Room	27.8
9	*B. vulgatus*	50	30	Room	33.0
9	*B. vulgatus*		37	37	43.2
9	*B. vulgatus*		50	50	56.0
10	*B. vulgatus*	50	30	Room	29.8
11	*B. subtilis*	30	30	Room	50.0
12	*B. subtilis*	30	30	30	50.2
13	*Asper. fumigatus*	30	30	Room	51.5
14	*B. subtilis*	30	30	30	54.1

*Unidentified.

temperature of the medium above 50.0°C. (from room temperature). Four of these are *B. subtilis*, one *B. vulgatus*, and one *Prot. vulgaris*. Three strains of what are apparently *B. vulgatus* were isolated which failed to show any appreciable heat-producing ability. The fact that most of the organisms which failed to heat upon cracked corn were isolated from heating cornmeal does not explain their lack of heat production, for two strains

which were inactive on the cracked corn were isolated from that same product. Comparisons between the temperature of isolation and the highest temperature produced show that organisms isolated at 30°C. may or may not heat to 50°, while strains isolated at 50° if they heat at all, will usually produce a temperature above 50°C. Studies have shown that the seeding of inoculum grown at 37° into corn which is then packed at room temperature usually results in very poor or no heat production. Further experimentation has shown that growth of the inoculum at 30° and packing and maintaining the inoculated corn at the same temperature gives the most reliable test of an organism's powers of thermogenesis.

The microbial flora present upon raw grains varies widely, and usually includes some molds. Since many molds can utilize the acids produced during bacterial metabolism, the possibility of symbiotic growth or interference arises. Removal of a portion of the acid may permit the bacterial species to produce still higher temperatures. In order to determine the possible effect of symbiotic growth, strains 2 and 13 (the latter, *Asper. fumigatus*, and the former, an unidentified rod-shaped bacterium) were packed into the same flasks (2) of corn and aerated with oxygen. The maximum temperatures produced, namely 53.2° and 52.8°, agree so closely with the maxima obtained with strain 13 alone and are only 6° higher than that obtained with strain 2 alone that the powers of thermogenesis of the latter strain can not be said to have been affected by the accompanying growth.

Culture 9 was found to be athermogenic when tested in the usual manner at 30°C. Abundant growth of the organism at 50° and poor growth at 30° suggested that the higher temperature might stimulate the strain to the production of heat. Corn inoculated with strain 9 which had been grown and packed at 50°, showed an insignificant rise of 6° in temperature (table 4). The possibility still existed that the organism would become actively thermogenic if stimulated by the heat produced by another organism. Therefore, strain 12 (former maximum 50.2°C.) was grown at 30° and inoculated into sterile corn which was packed into the lower thirds of two flasks, and strain 9 was grown at 50°

and inoculated into corn which was packed into the upper two-thirds of the same flasks. Heat produced by strain 12 (*B. subtilis*) in the bottom of each flask heated the upper contents to approximately the same temperature. The maximum temperatures produced, 53.2° and 51.8°C., approximate the maximum produced by strain 12 alone; hence no increase in thermogenic power can be attributed to strain 9 (*B. vulgatus*).

When the temperature of the above flasks had increased to the maximum of the *B. subtilis* strain inoculated into the corn in the lower layers, a larger portion of the oxygen introduced thereafter may have been consumed by this same organism, and but a small portion, if any, of it may have reached the *B. vulgatus* strain held in the upper part. Duplicate flasks were packed with the *B. vulgatus* in the *lower* two-thirds and the *B. subtilis* in the upper one-third. Maximum temperatures practically equal to those obtained previously were recorded, namely 52.3° and 50.0°C. The lack of evidence of a rise in temperature due to the activity of strain 9 apparently indicates a total absence of thermogenic power. This is by no means proven, however.

Several cultures reported above as being actively thermogenic, when later retested in the apparatus failed to produce any significant rise, and frequently produced no rise in temperature. Repeated tests confirmed these observations. This loss in thermogenic ability having been noted in strains which had been subcultured upon laboratory media for some weeks, the question naturally arose as to whether or not the organisms were still able to grow upon the corn substrate. Desiring to find some means of preserving this heat-producing power in order that it might be re-demonstrated at will, a strain of *B. subtilis* (14) was isolated from a mass of heating corn and subcultured weekly under the following conditions:

14W = cracked corn; subcultured directly.
14WH = cracked corn; water suspensions of the organism heated at 80° for twenty minutes and subcultured therefrom.
14WD = glucose agar; subcultured directly.
14WDH = glucose agar; water suspensions of organism heated at 80° for twenty minutes and subcultured therefrom.

Immediately after isolation the strain had produced temperatures around 53.0°C. repeatedly. Transfers made as described here were continued for six weeks and the thermogenic powers of the four sub-strains then retested. The results listed in table 5 show that strain 14WD (subcultured on glucose agar) was the only one which failed to produce maximal rises in temperature. Strain 14WDH (heated at 80° and grown on glucose agar) appeared still able to liberate considerable quantities of heat. The strains grown upon sterilized cracked corn were both actively thermogenic. The following conclusions appear warranted: first, cultivation from one tube of glucose agar to the next leads to the predominance of a so-called "weak" strain or sub-type

TABLE 5

Maximum temperatures produced by the same organism following six weeks subculturing upon sterilized corn and dextrose agar substrates

TEST NUMBER	DEXTROSE AGAR		STERILIZED CORN	
	Subcultured direct	Heated at 80°C. for 20 minutes	Subcultured direct	Heated at 80°C. for 20 minutes
	°C.	°C.	°C.	°C.
1	30.6	53.2	50.0	53.3
2	33.2	53.5	50.4	48.5
3	31.3	50.0	48.8	54.2
4	30.0	53.2	49.1	51.7
5	32.3	52.8	52.4	53.1
6			51.3	53.0

made up of cells able to utilize only the readily assimilable food substances; and second, subculturing in moist corn preserves the organism in a condition capable of showing marked heat liberation when tested by the technic used in these studies.

All organisms employed in the above pure culture experiments were isolated from some heating or heated raw material. The heat-producing powers of the following five laboratory organisms were tested in the usual manner: *B. cereus*, *B. mesentericus*, *Bact. coli*, *Bact. aerogenes*, and *Proteus vulgaris*. The inocula were produced on glucose agar and seeded in heavy suspension. No heating was observed by any of these organisms. Since these strains had been subcultured upon laboratory media for many

months their ability at any time to grow in cracked corn and cause heating is problematical.

CHEMICAL ASPECTS OF THE HEAT PRODUCTION

The studies referred to above have shown that carbohydrate substances furnish the greatest portion of the energy necessary for heat production. Boekhout and de Vries (1904) in a series of studies investigated the heating of hay from the chemical standpoint, concluding that, due to the high temperatures observed, microörganisms can play no part in the "spontaneous" heat production. After studying the origin of carbon dioxide found in large quantities in heating hay stacks, they concluded (1905) that the agent responsible for the decomposition is not soluble in water, 2 per cent hydrochloric acid or 2 per cent sodium hydrate. After artificially heating hay in different gas mixtures at 100° and at 33°C. they report (1908 and 1909a) that "spontaneous" heating is caused by oxidative processes which will act at 33° and more rapidly at 100°C. Iron is claimed to be an important catalytic agent. After studying tobacco fermentation the authors concluded (1909b) that there also the heating was due to an oxidation in which iron acts as a catalyzer. In order to remove the possibility of bacteria or enzymes playing any part in the heat production determinations were made of the carbon dioxide output from large test-tubes filled with normal hay, hay which had been heated at 56° to 60°C. for one-half hour, and hay which had been heated and then reinoculated with an infusion culture. This was reported in 1915. It was found that the tubes of unheated hay gave off much more carbon dioxide than the others. The authors state that if bacteria were the cause of heat production, the heated and reinoculated tubes should produce a large amount of carbon dioxide.

The last mentioned claim may or may not be justified for experiments have shown that the amount of inoculum introduced is an important factor.

Dry weights of unsterilized corn packed into several flasks compared with dry weights of the corn removed after the heating showed marked losses during the period of heat production.

The substance suffering this loss might well be claimed to be the source of the heat energy. The total carbohydrate contained in the corn packed and that in the material removed after the heating was determined by the method of Benedict (1911). The results of the analyses of three experiments are listed below:

Dewar A

	grams	grams
Dry weight of corn packed	157.45	
Dry weight of corn after heating	127.30	
Loss in weight during heating		30.15
Total carbohydrate before heating	118.75	
Total carbohydrate after heating	95.47	
Loss in carbohydrate during heating		23.28

Dewar B

Dry weight of corn packed	117.5	
Dry weight of corn after heating	88.8	
Loss in weight during heating		28.7
Total carbohydrate before heating	88.12	
Total carbohydrate after heating	67.48	
Loss in carbohydrate during heating		20.64

Dewar C

Dry weight of corn packed	153.7	
Dry weight of corn after heating	117.2	
Loss in weight during heating		36.5
Total carbohydrate before heating	116.8	
Total carbohydrate after heating	87.9	
Loss in carbohydrate during heating		28.9

In each of the above tests approximately 75 per cent of the loss in weight is accounted for by loss in carbohydrate; it must be assumed that the carbohydrate therefore furnishes most, if not almost all, of the energy liberated as heat. Since the heating of unsterilized cracked corn is accompanied by a marked depletion of carbohydrate substances the question arises as to whether or not the same would be true in the heating of pure cultures. Organism 12, an actively thermogenic *B. subtilis* strain was inoculated into cracked corn and packed into 3 different flasks. The maximum temperatures produced were 51.6°, 52.4°, and

51.8°C., respectively. On examination the corn in the flasks gave the following results:

Dewar A_1

	grams	grams
Dry weight of corn packed	101.45	
Dry weight of corn after heating	85.80	
Loss in weight during heating		15.65
Total carbohydrate before heating	73.45	
Total carbohydrate after heating	60.83	
Loss in carbohydrate during heating		12.62

Dewar B_1

Dry weight of corn packed	108.65	
Dry weight of corn after heating	93.40	
Loss in weight during heating		15.25
Total carbohydrate before heating	82.57	
Total carbohydrate after heating	69.00	
Loss in carbohydrate during heating		13.57

Dewar C_1

Dry weight of corn packed	229.90	
Dry weight of corn after heating	219.85	
Loss in weight during heating		10.05
Total carbohydrate before heating	167.82	
Total carbohydrate after heating	157.85	
Loss in carbohydrate during heating		9.97

In the above tests with pure cultures at least 80 per cent of the loss in weight was due to loss in carbohydrate. Thus it is shown not only that the bacteria in pure culture utilized the plant carbohydrate, liberating heat energy therefrom, but also that the effect upon the substrate was similar to that produced during the heating of unsterilized corn.

While the decomposition of carbohydrate was the outstanding physiological change occurring during the heating a certain amount of nitrogenous metabolism is also demonstrable. Total nitrogen determinations, upon normal and heated corn, were made by the Arnold-Gunning modification of the Kjeldahl method. No decrease of nitrogen was noted after the heating of either mixed or pure cultures (table 6). Considering that the acidity of normal corn is increased with carbohydrate decom-

position any ammonia produced would be fixed and therefore retained. The amount of ammonia produced was determined by the mild alkalinization of the heated corn followed by aeration into an acid solution of known strength. Immediately after removal from the apparatus the entire contents of each flask (originally 115 grams dry weight) was immersed in quarter-saturated sodium carbonate solution and aerated into decinormal hydrochloric acid for four hours. The nitrogen fraction of the ammonia collected in the acid solution was then readily determined.

TABLE 6

Total and ammonia nitrogen before and after "heating"

	TEST NUMBER	MIXED CULTURES (UNSTERILIZED CORN)		PURE CULTURES (STERILIZED, REINOCULATED CORN				
		Before "heating"	After "heating"	Before "heating"	After "heating"			
Total nitrogen (mgm. per gram of corn)	1	14.70	16.52		14.14			
	2	14.28	17.18		14.56			
	3	13.44	16.24		15.32			
Average		14.14	16.64		14.67			
					Hours incubation			
					36	40	65	85
Ammonia nitogen (mgm. per 115 grams of corn)	1	0.0		1.19	3.78	12.47	13.30	15.46
	2	0.8		0.70	10.22	12.74	22.16	28.14
	3	0.4		0.84				
Average		0.4		0.91	7.00	12.60	17.73	21.80

Ammonia-nitrogen determinations upon several lots of normal and heated corn are listed in table 6. Inoculation of sterilized corn slightly increased the ammonical-nitrogen content but subsequent growth of the organism accompanied by heat production liberated a much larger amount. The duration of the experiment markedly effected the total ammonia-nitrogen obtained, thirty-six hours producing an average of 7.0 mgm. and 85 hours an average of 21.8 mgm. per flask of corn.

SUMMARY

Moistened cornmeal and cracked corn heated to 62.+°C. in a specially designed apparatus, when aerated with oxygen. Moistened wheat and oats heated to a less extent under the same conditions. The heating of nine flasks of cornmeal and seventeen flasks of cracked corn, each filled with material of a different moisture content, showed that with both products about 30 per cent water was the most favorable for heating.

Bacteriological examination of meal at various stages of the rise in temperature showed an increase in the numbers of organisms growing at 30°C. to a point where the temperature had reached 50°; beyond that a marked decrease occurred. Large numbers of organisms growing at 50° appeared in platings made after the temperature of the meal had increased above 50°, though as the temperature became higher a decrease, rather than a further increase, was noted.

Continued aeration of two flasks of meal after they had reached their maxima, namely 62.+°C., resulted in maintaining temperatures above 60°, in one flask for four days and in the other for fourteen days, before they were dismantled. The former flask was still at 62.0° when removed, while the latter had cooled to 52.0° on the fifteenth day. The former appeared nearly sterile, while the latter contained a thermophilic mold in large amount. The H-ion concentration increased during heating from pH 6.0 to pH 4.2.

Moistened hay also heated readily in the apparatus, temperatures of approximately 60°C. being obtained. When packed into insulated 20-gallon crocks and aerated with oxygen, moistened cured hay and uncured alfalfa each heated to 60.+°C. Fresh stable manure packed into the crocks and aerated produced temperatures of 71.0 and 73.5°C., respectively, though no heating was obtained when oxygen or air was not introduced.

Single strains of organisms in water suspension inoculated into sterilized corn and aerated in the apparatus produced temperatures as high as 57.+°C. Twelve strains isolated from cornmeal and cracked corn were tested. Seven raised the tempera-

ture of the flask from 30° to 50° or above, and nine produced temperatures above 45.0°C.

The inability of certain organisms to produce heat in the apparatus could not be altered by accompanying growth of thermogenic forms.

Recently isolated strains subcultured upon glucose agar soon lost their power to raise the temperature of the medium. This was due to an inability to grow upon the cracked corn substrate. Strains maintained upon sterilized corn retained their heat-producing powers over the six weeks that they were tested. Five laboratory strains failed to show any heat production when tested in the apparatus, due probably to an inability to grow upon cracked corn.

Dry weight and total carbohydrate determinations upon six lots of heating corn (3 pure culture and 3 mixed culture experiments) before and after heating showed a marked decrease in dry weight during the heating, about 75 per cent of which was accounted for by the loss in carbohydrate.

The total nitrogen contained in the corn remained practically constant throughout the period of heat production. Ammonia nitrogen markedly increased, however, during the heating of both mixed and pure cultures, indicating that the loss in organic nitrogen was quite appreciable, though, as ammonia, the nitrogen was not removed from the flask.

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Note: The very interesting work of F. Hildebrandt, reported in the August 30th issue of the Centralbl. f. Bakt., Vol. 71 (1927), pp. 440–90, appeared since this paper was arranged and should be carefully reviewed by everyone interested in this subject.

THE PRESENT STATUS OF SYSTEMATIC BACTERIOLOGY[1]

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A review of the literature will show that the most popular term that has been used to describe systematic bacteriology is "chaos;" and this irrespective of the period of history under consideration. It had its beginning with Linné (1774) who, proposed the term *Chaos* as a generic term to be applied to those species of microscopic life that were so poorly known that their natural relationships could not be expressed clearly. From the time of Linné to that of the latest writer in the field of systematic bacteriology, the word has been overworked. We would challenge the accuracy of this term as applied to systematic bacteriology.

Can we prove our point when we accuse nature of being chaotic? Do we not know her as a most orderly mistress? Is it not that we have groped about in an orderly world shouting "chaos" when we ourselves were but ignorant and blind to the orderliness about us? It is our knowledge of the natural relationships and evolutionary development of this great group of living things that is chaotic, not nature. Moreover many of us are too indolent or too self-satisfied with our own field of research to learn even the principles underlying the science of taxonomy. Thereby we do but contribute to the existing confusion.

Since the time of Billroth and Cohn, bacteriologists have had a habit of interpreting bacteriological phenomena in one of two ways,—one that incautiously connects observed facts into a related whole, while the other demands impossible proof of relationship before accepting a theory as a working hypothesis. To a Billroth (1874), the staphylococci, diplococci, streptococci and

[1] Presidential address delivered at the twenty-ninth annual meeting of the Society of American Bacteriologists, Rochester, New York, December 29, 1927.

even rods found in pus were but stages in the development of a single type, his so-called *Coccobacteria septica*. To a Cohn, these were unrelated entities which were to be regarded as distinct species, at least until such time as a relationship could be proved.

As we have made progress, many early obscurities have been made plain; but we still have among us those who see proved stages in a life history in a "symplasm," or in structures which have a resemblance to gametes, oidia, etc. Contrasted to these we have those who, because the proof of these relationships is not immediately forthcoming, vigorously deny that structures of these types exist among bacteria. Meanwhile the cautious worker maintains an attitude of skeptical neutrality.

Some would so emphasize variation in the characteristics of bacteria as to deny the value of the species concept as applied to this group, stating that generations are passed through so quickly that one should not expect a sufficient fixity of characteristics to make it possible to recognize species. The recognition of life cycles, or of so-called "microbic diassociation" seems to these workers to upset completely the species concept as applied to bacteria. On the contrary, all proof of life cycles, variations and microbic diassociation, even if it be shown that it is possible to transform one so-called species of bacterium into another so-called species, does but give the systematic worker additional means of characterizing species. Was the classification of Coelenterates destroyed, when it was shown that many polyp forms were but the alternate generations of medusa forms previously assigned different scientific names? The term "cysticercus" applied to the larval stage of tapeworms is but a survival of the days when these bladder worms were thought to be parasites quite distinct from tapeworms.

Frequently those individuals who would deny the value of the species concept among bacteria would unhestitatingly accept this concept as something valuable and easily applied if only it is applied to higher plants and animals. I wonder if anyone who feels that it is difficult if not impossible to work out natural relationships among bacteria has ever tried to work out these relationships among such a group of individual animals as the

black bears of North America. I believe I am right in saying that specialists in this field have never agreed whether these animals should be placed in one, or in three or more species. The vines known generally as the blackberries of North America puzzle the best of our systematists. Those who know, tell me that if bacteriologists would but try their hands at some of these problems they would return to their cultures with a sigh of relief that among bacteria at least, characteristics show some fixity.

The test tube permits us to culture our organisms in a laboratory, and we can study a hundred or even a thousand generations in a few days time. Mere man has never found a way to study the direct application of the principles of heredity to elephants where a generation requires one hundred years. I much prefer to work with bacteria rather than elephants, especially in our overcrowded laboratory at Geneva.

I cannot understand why some still raise the specter of the ancient controversy over the use of morphological versus physiological characters in bacteriological classifications. Why should we care whether one or the other type of character is more useful in defining a genus? Or whether we should use one type of character in defining a genus and another in defining a species?

As Winslow (1914) stated in his address as the retiring president of this Society fourteen years ago, "There is no fundamental distinction between morphological and physiological properties, since all are at bottom due to chemical differences in the germ plasm, whether they happen to manifest themselves in the size and arrangement of parts or in the ability to utilize certain foodstuffs." Differences and resemblances in protoplasmic structure may not be visible under our microscopes; but they are none the less real.

If either the genus or the species represents a natural group of individuals, these individuals will show to the careful and persistent observer more than one character in common that distinguishes the group from all other groups, even closely related ones. Bacterial species, if they are to correspond to species in other groups of animals and plants, should show several distinguishing

characteristics, not one. It is for convenience only and because of inadequate knowledge that we tentatively accept bacterial species that have but a single distinctive character.

In spite of those who would criticize the use of physiological or habitat characters as a basis for classification, I feel that workers are entirely justified in tentatively assuming, for example, that the streptococcus of scarlet fever is a species distinct from the streptococcus of erysipelas even though it may be very difficult to find morphological or cultural characters upon which to base this distinction.

BACTERIOLOGICAL NOMENCLATURE

So much by way of introduction. The rules of biological nomenclature, drawn up as they are in codes, are as distasteful to the bacteriologist as are the rules of grammar to the schoolboy. We have absorbed Pasteur's scorn of all things of this type, forgetting that other able French micro-biologist, Raphael Blanchard, who as President of the International Commission on Zoölogical Nomenclature has done much toward securing an observance of the rules of binomial nomenclature and who has aptly compared these rules to the rules of grammar. However much we scorn grammar, I fear I should cause smiles on the faces of even this polite audience if I should use "Them is" or "I hain't got" in this address.

Yet many bacteriologists are content to make just as atrocious mistakes as these in their use of scientific names. Pasteur was at least accurate and consistent in his use of trivial names so that his writings are clear to those who understand French. Some of those who accuse others of being dry-as-dust grammarians when they ask for the observance of the rules of nomenclature deserve to have some fun made at their own expense when they clutter up their papers with a jargon composed of "V-P" and "M.R." tests, "R" and "S" forms, "T.B." "K.-L.'s" and "B. 1. a.", and the like.

In spite of the lone voice that pleaded for "Data is" in a recent note in *Science*, most of us agree that we shall continue *to try* to use "Data are," or to avoid the word altogether. If we agree

that we should abide by the rules of grammar, why are we not under the same obligation to observe the rules of the grammar of our own science? The difficulty is not so much lack of willingness as that most of us are ignorant of these rules. Until Buchanan (1925) reprinted the Codes of Nomenclature we did not have them in our bacteriological libraries and did not know where to secure them. Do not our teachers of bacteriology have an obligation at this place? Why have they not taught us, at least the principles of nomenclature? I suggest this as a topic to be considered at the round table discussion on the teaching of bacteriology that is to follow this address.

Law of Priority. The fundamental law of nomenclature is the "Law of Priority." The strict application of this law causes some splitting of hairs over what constitutes publication; and this cannot be avoided where authors are so ignorant of the proper methods of publishing new binomials. It is evident that the mere publication of a binomial, without the name being accompanied by a description sufficient to enable others to recognize the newly named species produces a *nomina nuda* that should be disregarded and never used subsequently.

It, however, frequently requires much study to determine whether these unsatisfactory descriptions are really such that the species cannot be recognized; and many subsequent workers, as indolent or as careless as the first author, prefer to assume that the earlier name should be discarded and so propose a name of their own, thereby increasing confusion.

Language to be used in publication. Then many descriptions are published in obscure places or in languages that we cannot read. The latter difficulty has greatly increased in recent years because of a development of a nationalistic spirit throughout the world. In recent months, original descriptions of species of bacteria have come to my desk printed in Russian and in Japanese; languages spoken by millions of persons who feel that they have just as good a reason for asking us to learn their language as we have for demanding that they learn ours. Can those nations that have done the chief work in the development of bacteriology maintain their viewpoint in the face of a world that ignores them?

Can we demand that descriptions of new species not published in Latin, English, French or German shall be regarded as invalid? I doubt it. The world grows smaller and the scientific progress of the future will not be so closely centered in a few nations of the earth as it has been in the past.

Exceptions to the law of priority on the basis of usage. The one place where exceptions can safely be made in the strict application of the law of priority as applied to bacteriological nomenclature is where usage has established a suitable name in the literature to the exclusion of a forgotten name. A close study will show that such cases are very rare, however. For American bacteriologists, it may seem quite evident that *Bacillus cereus* Frankland, 1887 is a generally accepted name for one of our commonest spore-forming rods and that the probably synonymous *Bacillus ellenbachensis* Stutzer, 1898 should be discarded. Yet to most European bacteriologists, the latter name seems so much better defined and so well established that it should not be discarded for *Bacillus cereus* on the ground of the priority of the latter name.

International commission on bacteriological nomenclature. Only a truly representative Court, or Commission on Bacteriological Nomenclature can settle such a difference in viewpoint in a way that will secure the greatest possible acceptance of the decision. Such a Commission is a well established and excellently functioning body among zoölogists. In spite of the disruption due to the World War, the very efficient secretary of this Commission, Dr. C. W. Stiles of Washington was able to maintain its work and bring it into active service as soon as international relationships were resumed. The function of the Permanent Committee on Botanical Nomenclature has been different from that of the Zoölogical Commission and its work has been seriously interfered with both by the war, and by death. Because the botanists meet in international congresses only once in five years whereas the zoölogists meet once in three years, the work of the botanists has always lagged behind that of the zoölogists. It has seemed at times almost impossible to make progress. Possibly the only way in which bacteriologists will be able to make progress will be by organizing their own international group.

Indefinitely described species. Let us return to the problem of the indefinitely described species, for this is a problem of great importance to bacteriologists. Other than those cases where the scientific name of a species is so poorly described that it is clear that it should be disregarded, there are many cases where the description is accurate enough to permit approximate identifications. Shall such names be discarded in favor of a new name because the old name is not clear cut in its meaning? In many cases, cultures of organisms have been preserved and studied by subsequent investigators who have established the characters of the species. In such cases, I believe the names of these species should be recognized even though later workers determine from these cultures, that the organism did not have the exact characters that were originally described. A good example of such a complicated case is found in the name *Escherichia acidi lactici* Bergey. The original work with this organism was that of Hueppe (1884). Although he studied the organism and he is given the credit by many authors for having suggested the name *Bacillus acidi lactici*, Zopf (1885) is the man who first published this Latin name for this organism. Now it happens that in those days, it was generally believed that all rod-shaped bacteria would form spores if only grown under the right conditions and Hueppe described his organism as a spore-forming rod. His cultures were, however, preserved and later investigations have shown that the organism should be placed in the colon group as a non-spore-forming rod. Under these conditions, the name can be preserved and used without ambiguity.

I believe that the majority of bacteriologists are sympathetic to the preservation of a name that has become familiar through long usage so long as no ambiguity has arisen in regard to it. The rules of nomenclature provide for the emendation of original descriptions by subsequent investigators provided these emendations follow well established rules as they have in the case cited.

Nomina conservanda. Either generic or specific names may be preserved as *nomina conservanda* under the terms of the International Botanical Code. Lists of these names (including no

bacteriological terms, however) were drawn up at the Vienna and Brussels Congresses, and our own Committee on Classification has proposed a list of 16 generic terms as *nomina conservanda*. Since the publication of the Congress lists, protest has arisen that many of the names were adopted without adequate study so that it is proposed to revise the list at the London Congress. The same criticism applies to the list of generic terms recommended by this Society, and there is little likelihood that the London Congress will recognize all of the names on our list. Even if the Congress in London does revise the list, there is little hope that it will be done in a judicial manner and produce a list of names satisfactory to all. Legislative bodies composed of delegates, many of whom are not experts in the laws to be applied, cannot render decisions that must be judicial in order to be acceptable. This work should be done, as in the case of Zoölogy, by an international commission composed of students of nomenclature which acts as a court of last resort.

Names that seem to their author more appropriate than any previously given. Because bacteriologists have normally approached their life work through the medical channel, or in recent years through physiological, chemical or biochemical channels, it is natural that some have raised the question why species cannot be named according to some such logical terminology as is used for chemical compounds where the name at once tells the nature of the sustance to which it is applied. The proposal is made by Orla-Jensen (1921) that names of bacteriological species shall be made to fit their natures as new knowledge is available. There is much sympathy for this viewpoint among those who find names such as *Escherichia* horrible to contemplate.

If living things were like chemical compounds, such an adjustable terminology would be splendid; but even the chemists are forced to resort to the law of priority in the case of the names of the elements, e.g., illinium vs. florentium (Noyes, 1927). The names given species of animals or plants are arbitrary designations like the names of the chemical elements, or our own names. While the names Smith, Carpenter, Cooper, and Thatcher were descriptive in the first place and were changed with changes in

occupation, we no longer discard them on this ground. Scientific names are supposed to be descriptive, but the authors of these names are not always fortunate in their choice of words.

Some object to names derived from proper names like *Serratia*, *Erwinia*, *Zopfius*, etc., but to those who are historically minded these names may have just as great meaning as *Aerobacter* or *Streptococcus*. Generic names formed from proper names are permitted, though not encouraged, in all codes of nomenclature, provided they are suitably formed. Bacteriologists also have the careless habit of using adjectives as nouns as in the generic terms *Fusiformis* and *Encapsulatus*.

The difficulties that arise when any investigator who pleases, feels that he has the right to disregard the law of priority because the name that some previous investigator has suggested does not appeal to him as properly descriptive is well illustrated by the situation in the genus of the red, chromogenic bacteria (a group that I fear has become a hobby with me). The present confusion of names arose primarily because Ehrenberg felt that the names given by earlier Italian investigators were not satisfactorily descriptive so he changed the name to *Monas prodigiosa* (1848), a name which signified that the organism was the flagellated animal which was the real cause of certain so-called miracles. We may pass over *ten* other generic names suggested for this organism, as few would hold today that any of them should be used, and confine our attention strictly to names advocated at the present time by active workers in the field of systematic bacteriology. Schmidt and Weis (1901) feeling that none of the names previously suggested really met the case proposed *Coccobacterium*, Orla-Jensen (1909) disregarded this name and suggested *Liquidobacterium* because these organisms were such active liquefiers. In 1920, Winslow and his associates feeling that *Erythrobacillus* was a more suitable name, and supposing that it had a priority that it did not have, suggested that this name be made a *genus conservandum*. Notwithstanding this abundance of names, Enderlein (1925) introduced bacterial names that suggest life history relationships and gave the name *Dicrobactrum* to the genus.

As the matter stands then, there are five generic terms now being advocated and in current use for this genus. There are those who, like ourselves, follow Buchanan and Bergey in accepting priority and so use *Serratia*. But each of the other groups feels that the name that they advocate is the most descriptive. How can we have anything but confusion if we accept the idea that a descriptive terminology is to be preferred to an arbitrary terminology? In this case, which is the most descriptive character—form of the cell, liquefaction of gelatin or red chromogenesis? Or, should the life cycle be regarded as more descriptive? After all, isn't a historic name also descriptive?

Proper form of bibliographical references. Systematists are sometimes accused of being name makers, who propose new names in order to get their own names attached to these new names. As if a Koch or a Pasteur did not know that, if he did something that was useful to humanity, he would get his name into print. Certainly professional men who are normal human beings work in a hope that they may win the approval of their fellowmen. Systematists appear to be quite human.

But the real reason why it is the custom to place the name of the author immediately after a binomial (without the comma between, please) is to prevent ambiguity and for bibliographical purposes. If given in a paper where the discussion is primarily on classification the reference should, in addition, include the date the name was given, page on which the name first appears in print, and a complete reference to the article should appear in the bibliography. The name of the author of the specific name is given in parentheses immediately after the binomial, followed by the name of the person who proposed the complete binomial. If either name is omitted it is the name in parentheses, regardless of the fact that this is usually the name of the person who first described the organism and who perhaps has studied it most completely. The important thing to a systematist is to be able to find the exact page on which a particular binomial was first printed.

There is a widespread belief among bacteriologists that the name of the person given in connection with a scientific binomial

should be the name of the discoverer, or at least of the person who made the first important study of the organism. Such a custom wastes much time for the person who is trying to discover the nomenclatorial status of a given binomial. At least this was my experience, when I became interested in the organism to which Bergey (1923) has given the name *Serratia indica*. In the *Manual* he naturally followed the custom of previous authors in attributing the specific name *indicus* to Koch. The tedious bibliographical search for an original description that does not exist, taught me some interesting bacteriological history but offered no other recompense. The facts proved to be that Koch isolated this red, chromogenic bacterium from the intestinal tract of an ape fed on cultures of cholera germs in the course of experiments made during his historic trip to Egypt and India in 1883–1884. The organism was smeared on paper and sent through the mails to the laboratory in Berlin. Because it survived the ineffectual fumigation of the time, it acquired an unjustified reputation for being resistant to disinfectants.

Koch may have written a scientific name on this culture when he sent it to Berlin; but it appears more likely that it acquired the specific name "*indicus*" in being passed about the laboratory during those momentous days. It appears certain that Koch did not publish a name for this organism until 1887 when he incidentally mentions it in the final report of the Cholera Commission. Here he gives it the name *Micrococcus indicus* placing it in the genus *Micrococcus* (although it is a distinct rod) apparently because he felt it was closely related to the organism then commonly known as *Micrococcus prodigiosus* Cohn.

Meanwhile, however, at least three workers associated with the Berlin laboratory, who had studied cultures of the organism, published descriptions of it in their textbooks, each one of them using the specific name "*indicus*." These were Eisenberg, Flügge and Crookshank. Apparently Eisenberg is the one who first published a binomial for the organism and it is his name that should be given with the binomial with the complete bibliographical reference as follows: *Bacillus indicus* Eisenberg, 1886, Bakteriologishe Diagnostik, p. 1.

The absurdity of crediting the name *Bacillus indicus* to Koch when he evidently preferred *Micrococcus indicus* as a name for the organism should be sufficiently evident. The custom of trying to pay tribute to previous workers for things they did not do becomes an exasperation, rather than an absurdity, when it causes one to waste valuable hours in hunting for bibliographical references that do not exist.

It may be interesting in passing to note that while there may be cultures of *Bacillus anthacis*, *B. subtilis* or of some other bacteria that have been carried in culture collections longer than cultures of *Serratia indica*, cultures as old as this cannot be numerous. The cultures of this red chromogen still show the characters that Koch noted and that caused him to regard it as something different from *Micrococcus prodigiosus*. Such cases seem to offer pretty conclusive support for a belief that there may be reasonable fixity of species among bacteria.

Date from which to start bacteriological nomenclature. Much discussion has taken place regarding the date that should be chosen as the starting point for bacteriological nomenclature. Zoologists have solved this difficulty by using but a single date, Linné, 1758. Botanists have, on the other hand, caused much trouble for themselves by selecting dates subsequent to 1753 (the date of Linné's most important botanical work) for some of the lower groups of plants, with which Linné did little or no work.

At the International Botanical Congress held in Vienna in 1905, a vigorous discussion took place over the date that should be selected as the starting point for bacteriological nomenclature. This debate tended to turn into a discussion whether Pasteur, Ehrenberg or Cohn was the founder of bacteriology. The net result was a postponement of the settlement of the question until the Brussels Congress in 1910. At this Congress action was again postponed. The interruption of international relations caused by the World War has prevented any further international action thus far.

Meanwhile our own Society has twice attempted to settle the matter for American bacteriologists and has really accomplished nothing. We have on our records two contradictory actions,

though some may hold that the second action repealed the first. The first action[2] was taken in 1907 in accordance with the suggestion of that able phytopathologist, Dr. Erwin Smith. At that time, the Society selected Cohn's work published in 1872 as the starting point for generic terms and 1881 as the starting point for names of species. The latter date was selected as the period when Koch introduced the use of liquefiable solid media as a means of getting pure cultures.

Ten years later when the majority, if not all of the members of the Society had forgotten the former action and Dr. Erwin Smith was no longer active in the Society, we adopted 1885 the date of Zopf's third edition of "Die Spaltzpilze" as the date for beginning all bacteriological nomenclature with the exception of a list of *genera conservanda* (Winslow et al., 1917). By misprint, this date is given as 1883 in the Final Committee Report (Winslow et al., 1920).

Recently[3] the bacteriologists present at the Ithaca International Botanical Congress (1926) recommended the acceptance of any name published since 1753 that is recognizable today. As a matter of fact, this does away with nationalistic jealousies and puts nomenclature on a sound basis. If Goodsir in 1842 described *Sarcina lutea* accurately enough for identification today, why pass over his work for that of a more recent worker? Dates later than 1753 are usualy selected in an effort to get rid of certain terms, that appear undesirable to those selecting the date; but all really undesirable terms are eliminated by the application of other rules of the Codes. No one can predict what viewpoint the Cambridge Congress (1930) will accept, but 1753 appears to be the most logical date that can be selected.

Type species concept. The most important modification of the rules of nomenclature in recent decades has been the general acceptance of the so-called type species concept. This has gradually won its way, first among the zoologists of the world, and then among botanists, chiefly the American and English botanists (Hitchcock, 1921).

[2] See descriptive charts issued by the Society of American Bacteriologists, 1907–1920.

[3] Proceedings not yet published.

Under this system, the genus is defined primarily by means of a type species rather than by means of a statement of the common distinguishing characteristics of the species in the genus. The genus then becomes that group of species which has been found, or may be found by future investigation to be closely related to the type species. The outstanding characteristics of this group of species are also named so far as they are known. Especially among bacteria where species are poorly known and new ones are being described all of the time, such a method of defining a genus leaves the limits of the genus indefinite and capable of adjustment, without confusion, to new knowledge as it is obtained. As defined in this way, genera have at least one fixed point,—the type species.

The advantages of this method of definition can be made plain by comparing briefly what has happened in the case of the two common generic terms *Bacillus* and *Bacterium*. The first of these terms was proposed as a generic term by Cohn in 1872 and was made to include *B. subtilis* (the hay bacillus), a so-called *B. ulna* whose identity is obscure, and *B. anthracis* (the anthrax bacillus). By the rules governing the selection of type species *B. subtilis* has become the type species of the genus. Because this is still a recognizable species, the term is fixed in meaning and it produced no real confusion when Migula (1900) and others redefined the genus so long as *B. subtilis* was retained as the fixed point in the genus. The term *Bacillus* may have a wider meaning to some than it has to others but it nevertheless may be used without serious misunderstanding of its meaning.

Bacterium, on the other hand, first proposed as a generic term by Ehrenberg in 1838 to include various indefinitely described species of rods, has completely lost its meaning because none of his species (including the natural type species, *Bacterium triloculare*) can be identified today. Subsequent workers have, therefore, selected any species they pleased as type, and they have pleased to redefine the term so many times and in such varied fashions that the only way the term can be used today without ambiguity is by defining it each time it is used.

Erwin Smith (1905) tried to fix its meaning by accepting

Cohn's definition of the term, making it equivalent to Migula's term *Pseudomonas*. Migula (1900), on the other hand, used it for non-motile rods of which he evidently intended *Bacterium anthracis* to be the type. Lehmann and Neumann (1896) have used it for non-spore-forming rods and this is a sense in which it is still generally used. The Committee of this Society, under the leadership of Winslow and following the suggestion made by Orla-Jensen, tried to fix it in their first report (1917) as the colon group, but found this unsatisfactory and so gave it a broader meaning in their final report (1920). In this they named *Bacterium coli* as the type species, a species quite different from those previously indicated as typical by Migula and by Erwin Smith.

Not only have the rest of us been asked by these masters in the field to systematic bacteriology that have been named, to accept these varied interpretations of the term, but many others have made similar requests. To Erwin Smith, the problem appeared like a simple dilemma—either discard the term or accept the first valid emendation. Since his publication, however, the dilemma seems to have grown into a whole flock of dilemmas with many horns or thorns, if such similes are permissible. All of the difficulty arises because Ehrenberg did not describe a single species in the genus that can be recognized today. The name is not fixed by anything. Consequently, it is a floater and a nuisance. We should discard it as Bergey had done, in favor of names that have definite meanings. Recent codes of nomenclature based on the type species concept make it obligatory to discard indefinite terms of this type.

Temporarily, however, it may be found useful to retain the term and use it as zoölogists have used the term *Vermes*, as a sort of dumping ground where species of non-spore-forming rods whose generic names are in dispute or that are poorly described and yet probably valid, may be placed. A similar suggestion was made by Breed, Conn and Baker (1918) several years ago but never found popular favor. It would, nevertheless, have saved Bergey's classification much criticism if the term *Bacterium* had been used to cover the inadequately described species included in *Achromobacter*, *Flavobacterium*, *Serratia* and related genera.

Such a policy would have saved the introduction of many new binomials that future investigators are certain to regard as invalid.

But let us return from this digression on the status of *Bacterium* to the type species concept. So important do the zoölogists regard this comparatively new method of defining genera that at their International Congress held at Budapest last September (Stiles, 1927) they made it a rule that beginning January 1, 1931, all descriptions of new generic terms must be accompanied by the designation of a type species or the name will not be regarded as valid. American botanists have already drawn up a so-called Type bases code (Hitchcock, 1919, 1921a) and various committees of our Society have expressed their approval of the practice of designating type species.

It is to the credit of American bacteriologists that they were among the first to recognize the value of the type species concept as applied to bacteriological classifications. To Buchanan (1916–1918) must go the credit of having been the first to apply this principle consistently throughout a classification of the group. The first Committee report was open to criticism because it failed to designate type species for all genera. This oversight was, however, corrected in the final report. Bergey has designated type species throughout his classification.

It is only necessary to refer to the criticisms of the Austrian bacteriologist, Janke (1926) and to that of Hall (1927) to show that some bacteriologists have failed to grasp the significance of this use of type species as a means of defining a genus. Both of these critics state that Bergey uses physiological or habitat characters as the primary means of defining genera, whereas the primary character of the genus is, in each case, that it is the group of species closely related to the type species, this statement being implied by the designation of a type species. Those who have attacked the Genus *Erwinia* (Winslow et al., 1917) on the ground that its designation as a group of peritrichic plant pathogens leaves it as a quite indefinite group have failed to realize that the designation (Winslow et al., 1920) of this genus as that group of species that are closely related to the type species *Erwinia amylovora* Committee makes it a very definite and unambiguous group.

As the type species concept has found its chief advocates in America, it is not surprising that European bacteriologists have failed to grasp the full significance of this newly applied method of defining genera. Thus, even those masters in the field of systematic bacteriology, Lehmann and Neumann (1927) although they use many of the same generic terms that are used by Bergey and other American bacteriologists, refuse to accept the name *Serratia* on the ground that they feel that Bergey's (and likewise Buchanan's) definition of the term makes it cover a heterogenous group of species. Our own investigations support the viewpoint that Lehmann and Neumann have expressed; but we do not feel that this gives us an excuse for discarding the term when a more limited definition (Breed and Breed, 1927) keeping the genus centered about the type species *S. marcescens* Bizio makes it an entirely satisfactory term.

CONCLUSION

In conclusion, it should be emphasized that nomenclature holds the same relationship to systematic bacteriology that grammar holds to literature. Classification, taxonomy, or systematic bacteriology are all terms applied to the expression of the evolutionary relationships of bacteria in a system of classification. While one must observe rules of nomenclature in order to talk in a language that shall be understandable to his fellow-workers in all parts of the world, yet it is evident that the real joy of the research worker is centered in trying to work out relationships of organisms.

It will always remain a pleasure to the able bacteriologist who is our President-elect, Miss Alice Evans, that her investigations (1923, 1925) revealed to her the close relationship between the so-called *Micrococcus melitensis* Bruce and the so-called bacillus of abortion described by Bang. Instead of belonging to the two widely separated genera indicated by the men who discovered these species, they are so much alike that the best of our investigators have had much difficulty in finding really satisfactory diagnostic characters. If any are inclined to question the practical value of academic studies of this type, let them consider the

practical applications of the information that Miss Evans secured as a result of her work.

But the value of such studies is even more evident when one reflects on the amount of printers ink that has been wasted in describing the cultural characters, physiology, serological reactions, or what not of Culture No. X and Culture No. 999. Such work remains almost valueless unless cultures are preserved (e.g., as in the case of Bacillus ϵ of Freudenreich) until the systematic relationships of the organism become clear and the species acquires a suitable name. It is doubtful whether anyone could successfully challenge the statement that the value of more than one-fourth of all published bacteriological work is seriously impaired because the authors of the papers have not appreciated the necessity of explaining the systematic relationships of the organisms studied, of giving the reader some idea of the authenticity of the cultures used or of preserving cultures.

It is not surprising that conservative thought was shocked into a great deal of useless criticism when students of systematic bacteriology felt it necessary to raise the cumbersome genus *Bacterium* to the status of a Family. This caused the introduction of many generic terms which, while many were not new, had never come into current use. Orla-Jensen (1909) began the process previously acknowledged by Kruse (1896) to be a necessity, by using 17 or more generic terms to cover the old genus *Bacterium*. Buchanan (1916–1918) followed with a similar action. The Committee of our Society was slightly more conservative than these authors in their preliminary report (Winslow et al., 1917), but in their final report (1920) increased the number of these groups of species taken from the old genus *Bacterium* to 20 or more. Meanwhile, Castellani and Chalmers (1919) quite independently drew up a classification with more than 17 genera taking the place of *Bacterium*.

But the storm of criticism brought on by the attempt of the systematists to make their classifications fit developing knowledge did not break in full force until Bergey (1923) ventured to recognize 31 of these genera, in addition splitting, as had the others, some genera other than *Bacterium*.

Those conservatives that protest against the mental effort involved in learning a series of unfamiliar names in place of the much over-worked *Bacterium* and *Bacillus* will settle back with a sigh of relief when they find that while Lehmann and Neumann in the 1927 edition of their classic Manual have followed the style of other systematists in breaking up the genus *Bacterium*, they have used only 14 scientific names in place of this term. Alas! however, for the peace of mind of those who wish for the simplicity of the good old times, they indicate the need for a dozen of more additional terms. The conservatives will however, find solace in the fact that they speak of the groups within the old genus *Bacterium* as subgenera and so continue to use this term in all of the binomials given for species in the genus.

On the whole, however, any person who makes a detailed comparison of these apparently dissimilar classifications must agree with Perkins (1928) (who has kindly allowed me to read a manuscript now in process of publication) when he points out "that on the whole, in spite of its defects, the work of the last twenty years has established sufficient recognized groups to enable us to develop these rationally without any complete upsets." Or again, "Not the least service of such attempts at complete classification of a series of organisms (concerning which the authors themselves are the first to admit our information deficient) is their courage in presenting their plans and ideas for criticism. Unless broad plans of this sort are accessible, revision is impossible. It is far easier to criticize than to construct, but only out of a combination of construction and constructive criticism can we hope to reach stability."

Please note that last word "stability." Here is an optimist who looks forward to the time when we shall have a much more definite idea whether there are only approximately a thousand species of bacteria, or whether they should be numbered by tens of thousands; and who sees that with increasing knowledge there will come increasing agreement even, as to the smaller groups in our systems of classification of the bacteria. A close comparison of the classifications that have been named will show that this optimism is based on solid grounds.

K. B. Lehmann in a personal communication received recently in which he expresses his appreciation at having been honored by election as a corresponding member of this Society speaks of the fact that systematic bacteriology is being properly emphasized in America. He adds the constructive suggestion that, while he has found it impossible to agree with some of the suggestions made in our recent classifications, he has refrained from any sharp criticism because he felt that it was very important to encourage investigations in this difficult field. If any are inclined to destructive criticism, they should consider the constructive attitude of this experienced investigator.

Much of the present confusion would never have occurred if we had been prosaic enough to have been willing to follow the well beaten paths that our colleagues, the zoölogists and botanists, have already trodden in their progress out of the wilderness of conflicting nomenclature. It is a paradox that we normally gain our liberties by obeying the laws, whether they be the laws of nature, or the laws of man. Much as we hate grammar, we gain liberty of action and the beauty of a great masterpiece by observing the rules.

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THE PROTEOLYTIC ACTION OF CERTAIN LACTIC ACID BACTERIA[1]

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Although the action of the lactic acid bacteria on carbohydrates has been the subject of many investigations, comparatively little attention has been paid to their proteolytic activity. Kulp and Rettger (1924) found that from 2 to 6 per cent of the proteins in milk were broken down by *L. acidophilus* and *L. bulgaricus*. Orla-Jensen (1919) studied the proteolytic action of a large number of lactic acid bacteria on the proteins of milk and Witte's peptone broth. In milk from 0 to 22.7 per cent of the total nitrogen was changed into soluble forms and in Witte's peptone the amino nitrogen produced varied from 0.5 to 33.9 per cent of the total. Ammonia nitrogen generally amounted to less than 2 per cent of the total nitrogen. Itano (1916) in studying the influence of hydrogen ion concentration upon the proteolytic activity of *S. lacticus* obtained an increase of 8.7 to 33.5 per cent in the amount of amino nitrogen. Sears (1916) reported that *B. acidi-lactici* produced much more ammonia and amino nitrogen when glucose was left out of the medium than when it was included.

Various criteria have been used by different investigators as indexes of proteolysis. The early investigators, Smith (1897) and Peckham (1897), took putrefactive products as a test, Heller (1921) employed pH changes, Hall (1922) used liquefaction of coagulated proteins and gelatin as standards, Kendall and Walker

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(1915) took liquefaction of gelatin and ammonia production as indexes, Itano (1916) employed changes in amino nitrogen and hydrogen ion concentration as measures and Berman and Rettger (1918) regarded the biuret test and changes in amino nitrogen as satisfactory criteria. Sears (1916), Waksman (1917), DeBord (1923) and Kendall, Day and Walker (1913) used both ammonia and amino-acid nitrogen determinations as methods for following protein decomposition. Kendall (1922), Kendall, Haner and Bly (1922) and Wagner, Dozier and Meyer (1924), used intermediate protein degradation products as well as ammonia and amino nitrogen as criteria of proteolysis.

In this paper the production of non-protein nitrogen and of amino acid nitrogen are used as main criteria of protein degradation, and ammonia formation is taken as an auxiliary index. Waksman and Lomanitz (1925) have pointed out that "Ammonia accumulation can serve as a good index of proteolysis only when no available carbohydrates are present; in the presence of the latter this method should be supplemented by the study of another process, either the disappearance of original protein, or the formation of amino nitrogen." It is obvious that more proteolysis takes place than is evident from analysis. The apparent proteolysis as determined by analysis is only the difference between production and consumption of the different forms of nitrogen.

EXPERIMENTAL

Media. Three different media, varying in their content of protein and glucose, were used, as follows:

1. One per cent glucose dissolved in yeast water. The yeast water contained about 0.1 per cent of nitrogen.

2. One per cent glucose and 1 per cent bacto-peptone in yeast water.

3. Two per cent glucose and 2 per cent nutrose (sodium caseinate) in yeast water. The nutrose was first made into a paste and then diluted with yeast water.

The yeast water was prepared as follows: Starch-free yeast was stirred with 10 times its weight of tap water until a smooth

suspension was obtained. After steaming the suspension for one or two hours, it was sterilized for two hours. After sterilization the suspension cleared rapidly. The clear liquid was siphoned off, and used for the yeast water media. After sterilization and inoculation, sterilized $CaCO_3$ was added and the cultures were incubated for various lengths of time at 28°C.

Cultures. The lactic acid bacteria used in this study have been divided into two groups; those that form mannitol from fructose and those that do not.

Of the mannitol-forming bacteria Cultures 19, 23, 26 and 36 were isolated from corn mash; Cultures 3-4, 4-14, 36-4, 36-6, 55-9, 57-8, 64-2, and 64-9, were obtained from sauerkraut and Culture 41-11 was found in soil.

In the non-mannitol group Culture A is a strain of *Lactobacillus leichmanni*, Culture 39-A is a strain of *Lactobacillus bulgaricus*, Culture 42 is a strain of *Lactobacillus delbrücki*, Culture R is a strain of *Streptococcus lactis* and Culture 40 is a strain of *Lactobacillus acidophilus*. Cultures 102, 25-7, 24-2 and 27-8 were isolated from sauerkraut.

The sugars fermented and the products formed by these cultures have been reported in previous publications from this laboratory (1920, 1922, 1925, 1927) and the results need not be repeated.

Fermentation. During the incubation period all cultures were shaken daily to bring about a better neutralization of acids by the calcium carbonate. The period of incubation was determined to some extent by the destruction of the sugar. Nitrogen determinations were not made until about 90 per cent or more of the glucose had been fermented.

Methods of analysis. At the end of the fermentation the cultures were diluted to their original volume and aliquots taken for the respective analyses. The unfermented sugar was determined by the Shaffer-Hartmann micro method as modified by Stiles, Peterson and Fred (1926).

Total nitrogen and non-protein nitrogen were determined by the Kjeldahl method. By non-protein nitrogen is meant the nitrogen contained in the filtrate of the aliquot after precipitation

with Folin and Wu's (1919) tungstic acid method. According to Hiller and Van Slyke (1922) tungstic acid is distinguished by the relative completeness with which it precipitates not only the native proteins, but also the protein intermediate products, without precipitating the amino acids.

The ammonia nitrogen content of the cultures was determined by the Folin aeration method as given by Hawk (1923). The aliquot 50 cc. of the culture was aerated in the presence of 2 grams of anhydrous sodium carbonate and 5 grams of sodium chloride for three hours. About 10 to 20 drops of methyl n-hexyl carbinol was used as an anti-foam. The removal of ammonia was much hastened by the addition of the sodium chloride. Tests were carried out on samples of known ammonia nitrogen content to find the speed of the air current and the time necessary to effect complete recovery of the nitrogen. Tests were also made to determine if the sodium chloride and anti-foam had any influence upon the amino nitrogen readings. It was shown that there was no such effect.

The ammonia content of the first set of cultures in table 2 was determined by Folin and Bell's (1917) permutit method. This method was discontinued and the above described aeration method was used in later experiments because it was found, as shown in table 5, that the permutit removed some amino nitrogen as well as ammonia.

The amino nitrogen was determined on the residue from the ammonia determination by means of Folin's (1922) colorimetric method. This method will determine 0.1 mgm. of amino nitrogen with an accuracy of 98 per cent or more and is therefore particularly well adapted to the analysis of cultures in which there has been but little proteolysis.

Proteolytic action on yeast water. Two hundred cubic centimeter portions of a 1 per cent glucose yeast-water medium were placed in 300 cc. Erlenmeyer flasks, inoculated with the various cultures and incubated at 28°C. for fourteen days. At the end of this time the different forms of nitrogen in the controls and in the fermented medium were determined. The results are given in table 1.

In series 1 all cultures showed an increase in ammonia and non-protein nitrogen and, with one exception, in amino nitrogen. The largest increases occurred in the non-protein fraction: from 30 to 40 mgm. more non-protein nitrogen were found in the cultures than in the control. Either the cleavage of the proteins

TABLE 1

Proteolysis in glucose yeast water medium

Non-mannitol formers

CULTURE NUMBER	GLUCOSE FERMENTED	NON-PROTEIN-NITROGEN	$NH_3 - N$	$NH_4 - N$
	Series 1. Incubated 14 days			
	mgm. per 100 cc.	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
Control*	981†	53.8	20.0	2.4
40	942	92.6	21.2	5.2
R	945	95.2	20.1	5.8
102	933	95.6	21.5	4.5
A	911	93.0	23.8	5.5
24-2	945	87.2	20.6	4.8
27-8	888	94.8	21.4	3.8
39-A	901	93.2	22.0	5.6
25-7	931	84.3	18.0	5.4
	Series 2. Incubated 18 days			
Control‡	1,680†	60.4	28.6	1.6
42	1,637	83.8	35.7	3.0
R	1,627	74.1	29.4	5.6
A	1,281	74.9	37.9	2.4

* Control and all cultures contained 108.0 mgm. of total nitrogen per 100 cc. of medium.

† Glucose in the unfermented control.

‡ Control and all cultures contained 104.7 mgm. of total nitrogen per 100 cc. of medium.

was very slight, since there is very little increase in the quantity of amino nitrogen, or the consumption of amino acids approximately equalled the production.

In series 2 the percentage of sugar was greater and the incubation period longer. The extent of proteolysis was somewhat greater in this case. The ratio of amino nitrogen to non-protein

nitrogen was greater than in the first series. Also the quantity of amino nitrogen produced was greater than in series 1.

Proteolytic action on peptone yeast-water. A larger number of cultures were used in this experiment than in the two preceding

TABLE 2

Proteolysis in peptone-yeast water medium

CULTURE NUMBER	GLUCOSE FERMENTED	TOTAL NITROGEN	NON-PROTEIN-NITROGEN	$NH_3 - N$	$NH_2 - N$
	Mannitol formers, incubated 10 days				
	mgm. per 100 cc.	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
Control	975*	252	139		4.4
57-8	926	252	161		7.9
64-9	918	252	185		6.0
64-2	921	252	206		3.5
36-6	920	252	204		4.1
55-9	926	252	206		3.2
36-4	925	252	145		5.7
Control	975*	266	134	37.1	4.4
4-14	936	266	154	51.6	6.6
36	936	266	151	52.7	4.3
41-11	956	266	148	50.7	6.9
19	936	266	147	52.7	1.4
23	945	266	146	49.8	5.7
3-4	917	266	147	35.8	1.9
	Non-mannitol formers, incubated 14 days				
Control	985*	223	109	50.5	2.0
39-A	919	223	155	62.9	3.1
A	941	223	129	50.8	1.8
40	476	223	142	53.2	1.0
R	939	223	171	66.2	2.2
102	937	223	179	74.6	1.8
25-7	940	223	174	71.9	2.4
24-2	936	223	153	57.8	1.9
27-8	939	223	168	52.0	1.4

* Glucose in the unfermented control.

ones. Two groups of mannitol formers and one group of non-mannitol formers were tested for proteolytic activity. Judged from the point of view of sugar destruction, good fermentations were obtained with all but one culture, No. 40. In this case only

about one-half of the sugar was destroyed and correspondingly small changes in the forms of nitrogen were observed. The data are given in table 2.

The medium in this experiment contained about two and one-half times as much nitrogen as in the experiment of table 1. As a consequence there was a considerably larger increase of the various cleavage products. As in the first two experiments, the

TABLE 3

Proteolysis in glucose nutrose yeast water medium

Mannitol formers, incubated 6 to 11 days

CULTURE NUMBER	GLUCOSE FERMENTED	NON-PROTEIN NITROGEN	$NH_3 - N$	$NH_3 - N$
	mgm. per 100 cc.	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
Control*	1,780†	70.6	45.4	3.1
57-8	1,727	70.6	29.9	5.0
64-9	1,742	61.1	26.0	3.6
64-2	1,742	71.4	23.7	3.3
36-6	1,742	64.5	22.8	2.7
55-9	1,742	62.0	36.8	3.0
36-4	1,727	66.4	35.2	5.2
4-14	1,709	71.1	20.6	3.1
36	1,742	63.6	27.0	4.4
41-11	1,709	67.2	37.7	4.8
19	1,742	79.4	34.5	5.3
23	1,742	63.6	30.2	3.2
3-4	1,742	62.0	24.0	2.7
26	1,742	60.3	28.5	4.1

* Control and all cultures contained 317 mgm. of total nitrogen per 100 cc. of medium.

† Glucose in the unfermented control.

non-protein nitrogen shows the largest increase. In some cultures the increase is from 60 to 70 mgm., and in such cultures there is a corresponding increase in amino nitrogen. The increase of ammonia nitrogen is in no case more than 3.5 mgm. and in a number of cases there is an actual decrease. The principal products of proteolysis were evidently proteoses, peptones and amino acids rather than ammonia.

The data show no particular difference in the proteolytic action of the mannitol formers and non-mannitol formers. Both

groups of microörganisms produced about the same increases in non-protein nitrogen and amino nitrogen. The ammonia production of the mannitol formers was slightly greater but the increase was not significant.

No amino nitrogen figures are given for the first group of mannitol formers. The ammonia in this group was determined by absorbing with permutit, liberating with sodium hydroxide and Nesslerizing. It was later found that permutit also absorbed amino nitrogen and as a consequence the data which had been obtained on the ammonia residues were too low and had to be discarded. In all of the other cultures of table 2 the ammonia was determined by Folin's aeration method. The residues from this analysis proved perfectly satisfactory for amino nitrogen determinations.

Proteolytic action of mannitol-formers on nutrose yeast-water. Both glucose and nitrogen were increased in this experiment. A more complex form of nitrogen, nutrose, was used. That a good fermentation was obtained is indicated by the almost complete destruction of the glucose.

From the data in table 3 it is difficult to decide whether the bacteria attacked the nutrose or did not attack it. Both the non-protein and amino nitrogen decreased in amount; the latter more than the former. It appears as if the bacteria used the simpler forms of nitrogen in the yeast water and left the nutrose untouched. There is no certainty about this conclusion, however, since the forms of nitrogen measured merely represent the difference between production and consumption. When there is an increase, the evidence is conclusive; but when there is no change or a decrease, no definite conclusion can be drawn. In the large majority of cases the different forms of nitrogen decreased in amount.

The failure to get any evidence of proteolysis may have been due to the short incubation period. As will be shown in the next experiment, there is a time factor which seems to be more important in the breaking down of the proteins than in the destruction of the sugar.

Proteolytic action of non-mannitol formers on nutrose. The

medium was the same as that used for the mannitol formers but the incubation periods were about two or three times as long.

The data in table 4 show that most of the cultures brought about considerable proteolysis. The non-protein nitrogen de-

TABLE 4

Proteolysis in glucose nutrose yeast water medium

Non-mannitol formers

CULTURE NUMBER	GLUCOSE FERMENTED	NON-PROTEIN-NITROGEN	$NH_2 - N$	$NH_2 - N$
	Incubated 20 days			
	mgm. per 100 cc.	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
Control*	1,618†	79.0	26.3	2.8
39-A	1,543	103.9	48.1	4.6
A	1,543	126.5	45.5	4.0
40	1,543	89.4	36.5	4.4
R	1,563	57.2	32.3	3.8
102	1,562	87.0	25.3	3.0
25-7	1,537	67.7	28.1	4.4
24-2	1,555	85.4	28.8	3.4
27-8	1,560	130.5	25.3	4.0
42	1,563	107.1	59.5	4.8
	Incubated 39 days			
Control*	1,618†	78.6	26.2	2.6
39-A	1,543	148.2	39.2	5.6
A	1,542	148.2	47.1	7.3
40	1,547	167.6	40.8	6.0
R	1,565	96.7	28.2	6.7
102	1,567	112.8	26.8	4.2
25-7	1,549		29.2	4.8
24-2	1,565	122.5	30.1	3.8
27-8	1,566	109.6	28.4	3.8
42	1,563	161.1	46.5	5.6

* Control and all cultures contained 326 mgm. of total nitrogen per 100 cc. medium.

† Glucose in the unfermented control.

creased in 2 cases but increased in 7 others and averaged 20 per cent more than the control for all the cultures. Amino nitrogen increased in 7 out of 9 cases and showed an average increase over

the control of 39 per cent. Ammonia nitrogen increases ranged from 7 to 71 per cent and averaged 44 per cent.

In the longer period of incubation, thirty-nine days, all cultures showed increases for each form of nitrogen. The increases for

TABLE 5

The effect of the removal of ammonia by permutit on the determination of amino nitrogen

SOLUTIONS ANALYZED	CONCENTRATION	TREATMENT	$NH_3 - N$	$NH_3 - N$ REMOVED
	per cent		*mgm. per 100 cc.*	*per cent*
Yeast water	10.0	Aeration*	29.2	
		Permutit	20.6	29
Combined peptone + beef extract	0.5 + 0.3	Aeration†	58.8	
		Permutit	48.4	18
Combined yeast water + peptone	10.0 + 1.0	Aeration‡	50.7	
		Permutit	38.3	24
Glutamic acid	0.0142	None	1.35	
		Permutit	0.90	33
	0.0284	None	2.70	
		Permutit	1.36	50
	0.0568	None	5.40	
		Permutit	2.86	47
Histidine	0.0173	None	1.56	
		Permutit	0.68	56
	0.0346	None	3.12	
		Permutit	0.98	69
	0.0691	None	6.24	
		Permutit	1.44	77

* 2.4 mgm. $NH_3 - N$ per 100 cc.
† 0.8 mgm. $NH_3 - N$ per 100 cc.
‡ 2.0 mgm. $NH_3 - N$ per 100 cc.

the three forms were as follows: non-protein nitrogen from 23 to 113 per cent, amino nitrogen from 2 to 80 per cent and ammonia nitrogen from 46 to 180 per cent.

The results from these two series leave no doubt that nutrose can be broken down by the non-mannitol forming bacteria. The data for sugar show that practically all of the glucose was destroyed in twenty days. From the results in tables 2 and 3 it seems probable that most of the glucose had disappeared in ten days. Proteolysis continued even though there was no more glucose to be fermented. From these data it appears that fermentation is much more rapid than proteolysis. The two processes do not seem to parallel one another. Perhaps the slight proteolysis of nutrose obtained with the mannitol-formers was due to insufficient time and not to inability to attack the protein.

The removal of ammonia from cultures by means of permutit. Some indications had been obtained in the previous experiments that permutit not only combined with the ammonia in the culture media but also removed some amino nitrogen. In order to obtain more definite information on this point three kinds of culture media were analyzed for amino nitrogen before and after treatment with permutit. Folin's (1922) procedure for the removal of ammonia from urines to be analyzed for amino nitrogen was followed. The concentrations of amino nitrogen chosen were approximately those reported by this investigator for human urines. The results are given in table 5 and show that from 18 to 29 per cent of the amino nitrogen in the media was removed by the permutit. These results are in agreement with the findings of DeBord (1923) who reported that permutit was unsuitable for the removal of ammonia from bacterial cultures.

Whitehorn (1923) has shown that the substances removed from solution by permutit are relatively strong bases. It is probable that permutit removes both peptides and amino acids. Two amino acids of different basicity, glutamic acid and histidine, were tested with permutit. Solutions containing varying concentrations of these amino acids were treated with permutit and the amino nitrogen determined before and after treatment.

The permutit removed more histidine than glutamic acid, which is in accordance with the basicity of the two compounds. The percentage removed increases with the concentration. In

the case of histidine, regardless of the absolute quantity present, everything in excess of about 1 mgm. of amino nitrogen per 100 cc. was taken out. As a result, in some cases more than 75 per cent of the total amino nitrogen was removed from the solution. Permutit obviously can not be used to remove ammonia from culture media which are to be analyzed for amino nitrogen.

SUMMARY

The proteolytic action of 22 strains of lactic acid bacteria has been studied. Of the 22 strains, 13 were mannitol formers and 9 were non-mannitol formers. Three different kinds of media varying in nitrogen content from 105 to 326 mgm. per 100 cc. were used. Formation of non-protein nitrogen, amino nitrogen, and ammonia nitrogen was taken as a measure of proteolysis.

Non-protein nitrogen was the most abundant form of nitrogen produced. In a few cases there was a slight decrease but in general there was an increase of this constituent. The maximum increase occurred in a nutrose yeast-water medium containing 326 mgm. of total nitrogen per 100 cc. In this medium 89 mgm. of non-protein nitrogen per 100 cc. were produced. Including the preformed non-protein nitrogen of the control, this constituent amounted to about 51 per cent of the total nitrogen.

In some cases more amino nitrogen was consumed than was produced. The variations ranged from a decrease of 24.8 mgm. to an increase of 33.2 mgm. per 100 cc. of culture.

Ammonia production was small in all cases and in some cases did not equal its consumption.

Proteolysis continued long after destruction of sugar had ceased.

No marked difference in the proteolytic action of the two groups of lactic acid bacteria was observed.

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A THERMOPHILE COAGULATING MILK UNDER PRACTICAL CONDITIONS

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INTRODUCTION

Organisms capable of growing in milk at unusually high temperatures have interested various dairy bacteriologists because of their occasional growth in pasteurizing equipment. For the most part these organisms have caused difficulty through their responsibility for excessive bacterial counts rather than through any change in the milk itself. Many of them evidently grow at the temperature used for making plate counts on milk and accordingly are not strict thermophiles.

The Iowa Agricultural Experiment Station was recently asked to study a change occurring in milk held at a high temperature in one of the plants of the state. The surplus skim milk was being dried and, because of the small capacity of the drying equipment, it was necessary to hold some of the milk for a considerable period, sometimes up to twenty-four hours. This milk was pasteurized at from 71° to 77°C. and then run hot into a wooden tank where it was held; it was drawn from the tank as required to keep the drying equipment in operation. Before all the skim milk could be dried the portion remaining in the wooden tank occasionally coagulated and since the temperature fell slowly, due to the large volumes and the delayed heat transfer through the wood, this occurred when the milk was still quite hot.

EXPERIMENTAL

Two samples of normal pasteurized skim milk from the wooden tank were packed in ice and forwarded to the laboratory, where

some of the milk was transferred aseptically to sterile test tubes plugged with cotton and then held in an incubator at 71°C. Coagulation occurred in about eighteen hours, while both skim and whole milk secured in Ames and kept under the same conditions failed to coagulate even after seventy-two hours, which was as long as they were held. The samples of skim milk sent to the laboratory were kept packed in ice and the tests at 71°C. occasionally repeated; coagulation occurred regularly in about the time required for coagulation in the first trial.

Microscopic examination of the milk which had coagulated at 71°C. showed the presence of Gram-positive organisms in rather small numbers; they had the appearance of being in pure culture. Tubes of sterile milk inoculated with the coagulated milk usually coagulated in fifteen hours when held at 71°C., but showed no change at 37°C. or room temperature. The developed transfers showed organisms of the same general morphological characters as those in the original coagulated milk.

Plates were poured for isolation using standard beef extract agar but those held at 37°C. or room temperature showed no growth and those held at 71°C. quickly dried out, although an attempt was made to keep up the humidity of the incubator. Some of the coagulated milk was then smeared on the surfaces of standard beef extract agar slopes containing 1 per cent glucose and the slopes held at 71°C.; in about twenty-four hours thin, white, rather small colonies were evident and these showed organisms microscopically resembling those present in the original coagulated material. When organisms from the colonies were transferred back to sterile milk and this held at 71°C., coagulation quickly occurred. Various attempts were made to isolate organisms by smearing coagulated milk on agar slopes and holding at 71°C. but only one type of organism seemed to develop.

In litmus milk at 71°C. the isolated organisms gave a smooth curd with a reduction of the litmus in the lower layers so that there was a pink band at the surface. The general appearance was much like that of litmus milk cultures of *S. lactis*, although the pink band was never so narrow as it often is with freshly

coagulated *S. lactis* cultures, and it quickly increased in width. Even when young cultures were removed from the 71°C. incubator and held at room temperature the pink color soon returned to the milk; this was presumably due to the fact that at the lower temperature the growth of the organisms quickly stopped and the oxygen of the air then brought back the color in a short time.

Titrations made on plain milk which had coagulated after inoculation with the organism gave acidities of from 0.40 to 0.45 per cent, calculated as lactic acid. Since such acidities do not ordinarily result in the coagulation of milk at room temperature of 37°C., it seemed advisable to determine whether the coagulation at 71°C. was due to acid or to a coagulating enzyme. The procedure followed was to inoculate *S. lactis* into milk, allow it to grow at room temperature and then, before enough acidity had developed to cause coagulation, place the tube of milk in a beaker of water which had been held for some time in the 71°C. incubator. Milk which had not coagulated at room temperature sometimes coagulated when quickly raised to 71°C. by placing it in the water; since the ordinary *S. lactis* cultures are rapidly killed at this temperature, it does not seem that there could have been any appreciable increase in acidity after the temperature change. The tubes of milk which coagulated when transferred to 71°C. were titrated and the lowest acidity found accompanying coagulation was 0.39 per cent while acidities slightly above 0.40 per cent were repeatedly found. It accordingly seems that the coagulation at 71°C. with the isolated organism is due to the acid produced, although the amount of acid present is lower than that usually considered necessary for coagulation at ordinary temperatures.

Since the organism isolated is so different from the acid producing organisms usually found in dairy products an attempt was made to secure information on the nature of the acid developed. Fermented milk was subjected to the procedure used at this station (Iowa, 1920 b) for the determination of the type of lactic acid produced in milk; the data obtained on two preparations of

the zinc salt are given in table 1. The moisture values are close to the 12.88 theoretical for the active lactates and the ZnO percentages agree with the 33.46, theoretical for the anhydrous salt. The rotation of the lactate was *l* which corresponds to *d* lactic acid.

The volatile acid production of the isolated organism was determined by the method in use at this station (Iowa, 1920 a); it was found in a number of trials to be very low with the volatile acid making up but a small per cent of the total acidity.

TABLE 1

Results on the zinc salts prepared from milk fermented by the organism isolated

TRIAL	INCUBATION OF FERMENTED MILK STUDIED	PER CENT H_2O OF CRYSTALLIZATION			PER CENT ZnO	ROTATION
		Determination A	Determination B	Average		
1	4 days at 55°C.	13.47	13.26	13.365	33.68	l
2	4 days at 55°C.	13.25	13.13	13.19	33.54	l

TABLE 2

Action of isolated organism on nitrogenous constituents of sterile milk

MATERIAL ANALYZED	AGE OF CULTURE	PER CENT OF TOTAL N AS SOLUBLE N
	hours	
Uninoculated milk no $CaCO_3$		10.08
Uninoculated milk with $CaCO_3$		9.82
Fermented milk no $CaCO_3$	40	10.08
Fermented milk no $CaCO_3$	96	11.20
Fermented milk with $CaCO_3$	40	11.52
Fermented milk with $CaCO_3$	96	12.75

The action of the organism on the nitrogenous constituents of sterile milk was studied as follows: Milk in 200 cc. quantities was put into pint bottles and to some of them 8 grams $CaCO_3$ and some pieces of glass were added; after stoppering with cotton and sterilizing in the autoclave the bottles were allowed to cool and then inoculated. During the holding period the bottles containing the $CaCO_3$ and glass were frequently shaken to bring

the $CaCO_3$ in contact with the acid formed. The soluble nitrogen was determined by making up the lost H_2O, adding 1 cc. of glacial acetic acid per bottle, heating to 60°C. with occasional shaking and then cooling and filtering; Kjeldahls were run on 25 cc. quantities of whey. The results secured, which are given in table 2, suggest a slight increase in the amount of soluble nitrogen, especially with $CaCO_3$ added, but the decomposition is only very small in amount. The action of the organism on the protein of milk is more like the action of *S. lactis* than that of the typical spore forming organisms.

The temperature range over which the organism will grow was studied, using milk as the medium since it grows better in this than in any other material tried. At 45°C. slight growth sometimes occurred but not always. The maximum temperature used was 75°C. and coagulation occurred rapidly, but apparently not quite as rapidly as at 55° to 65°C.

While the original attempts to secure coagulation by holding milk obtained in Ames at 71°C. gave negative results, later trials were positive. The organisms present in this coagulated milk were rods that could be carried along through a series of transfers. However, these organisms also grew at 37°C. and in this respect were quite different from the organism found in the milk forwarded to the laboratory from the plant experiencing trouble with coagulation at a high temperature. Moreover coagulation was slower at both 37° and 71°C. than with the true thermophile at 71°C. and was followed by a very evident digestion instead of yielding a typical acid coagulation.

A thermophile which grew at lower temperatures in soil than on agar or in bouillon was reported by Koch and Hoffman (1911) and accordingly an attempt was made to determine whether or not the isolated organism would grow in soil at 37°C. Because of the difficulty of inoculating the soil uniformly, counts did not seem advisable and the procedure used was to add sterile milk aseptically to a flask containing sterile soil until the surface of the milk was slightly above the soil, inoculate and then incubate at 37°C. The milk did not coagulate even after long holding

periods but it coagulated quickly when the flask was later exposed to a temperature of 55°C. The saturation of the soil may have influenced the growth of the organism so that the results are not entirely comparable to those of Koch and Hoffman.

Although the isolated organism would not grow at 37°C. or room temperature, it remained viable for a long time in milk since transfers made from cultures held at these temperatures to milk quickly gave typical rapid coagulation when the milk was held at 55°C. The organism also survived for a considerable period in a cooler which was being run at from about 0° to 8°C. These results suggest that the organism can readily live over periods during which it is not subjected to the high temperature apparently necessary for growth; they also indicate how the organism might be spread by the transfer of various materials in which it was present in a dormant condition.

IMPORTANCE OF THERMOPHILIC RODS IN DAIRY PRODUCTS—HISTORICAL

A number of investigators have studied thermophilic organisms and have isolated them from various materials, including certain dairy products. They are of many different types and are grouped in various genera. For the most part they have not been responsible for any abnormal condition in the materials from which they have been secured but are simply present and can be isolated by the proper technique. Morrison and Tanner (1922) have reviewed these organisms in considerable detail.

Fay (1927) has presented a review of the literature dealing with "pin-point" colonies in plates poured for determining the numbers of bacteria in milk and the relationship of various organisms to these. In his own studies this investigator found that the many forms of thermophilic spore-bearing rods isolated would not produce "pin-point" colonies when cultured at 37°C.

Observations which are of considerable interest from the standpoint of the work herein reported have been made by Mr. C. E. Gray of the Golden State Dairy Products Company of

San Francisco. These are outlined in the following abstracts from a letter received from him under date of April 15, 1927.

Rather early in our activities in milk drying we found that there were certain economies coming from holding milk at high temperatures after pasteurization, and so far as we were able to see there were no ill effects. We began by holding milk at 145°F. and at this temperature I felt quite sure that there would be no possible bacterial growth; in fact, all the literature up to that time showed that the number of living organisms decreased when milk was held at this temperature. However, after a time we found that the acidity was going up and the casein actually coagulating. My first thought was that errors were being made in taking the temperature. However, a careful investigation showed conclusively that the milk was at a temperature of 145°F. in all parts of the vat. Samples of this milk were taken and by making transfers to sterile milk and incubating at 145°F. what appeared to be pure cultures were very readily secured. . . . .

What we did was to raise our holding temperature to 160°F. and the milk then remained without acid development. I am not able to give you the exact date when this occurred but believe it to have been about 1912. Since that time the existence of organisms growing at high temperatures has been generally recognized and, as you know, the matter is of considerable economic importance, especially in city milk pasteurization plants.

Since that time we have established a number of dry milk plants in other parts of California and in practically all of them we have more or less trouble with the organisms growing at high temperatures. However, where the entire body of milk is held at 160°F. we believe it is rather a rare thing for acidity to develop. Where acidity has developed we have been inclined to attribute such development to a failure to keep all parts of the vat of milk up to 160°F.

CHARACTERS OF THE ISOLATED ORGANISM

The organism isolated seemed to be different than the thermophiles described in the literature. The combination of characters shown by it is very unusual. It is a strictly thermophilic spore bearing rod which produces the same general type of change in litmus milk that the true lactic acid organisms such as *Streptococcus lactis* and *Lactobacillus casei* produce, including the coagu-

lation due to the development of lactic acid, the reduction of the litmus and the comparatively small effect on the protein.

The organism is believed to be a new species and the name *Bacillus calidolactis* is proposed for it. The description follows:

DESCRIPTION OF THE BACILLUS CALIDOLACTIS

Morphology

Form. The organisms were rod shaped with some cells slightly curved; these were especially noticeable in old agar cultures.

Size. Organisms from an agar slope grown at 55°C. varied in length from 2.6 to 5.0 microns and in width from 0.7 to 1.4 microns. Organisms from milk showed essentially the same dimensions.

Arrangement. The organisms in milk or on agar incubated at 55°C. usually occurred singly although pairs and short chains were not uncommon.

Motility. The organisms from 18 hour agar or glucose broth cultures at 55°C. were non-motile.

Staining. The cells in young agar or milk cultures were practically all Gram-positive. As the cultures grew older Gram-negative cells appeared and the number of these increased rapidly as the culture aged.

Spore formation. Oval, terminal or subterminal spores that caused a slight swelling of the cells were present in forty-eight hour glucose agar cultures; the number was always small. Spores were never seen in milk cultures.

Cultural characteristics

Agar slopes. At 55°C. no growth was secured on either whey or standard agar slopes while 1 per cent glucose agar slopes (pH 7.2) showed an abundant, echinulate, rather dull, white growth after 16 hours incubation. This increased somewhat as the culture aged.

Agar stabs. At 55°C. growth was questionable in both whey and standard agar stabs. With 1 per cent glucose agar stabs an abundant, rather beady, gray growth was evident, after sixteen hours, along the entire length of the stab. Some stabs also showed a surface growth.

Agar plate colony. When incubated at 55°C. no growth was secured on either whey or standard agar plates while 1 per cent glucose agar gave abundant growth in sixteen hours. The surface colonies were thin, white, opaque, with filamentous edges, and were about 1 mm. in

diameter. The subsurface colonies were white and irregular and had a compact, dense center and filamentous edges; they were about one-third as large as the surface colonies.

Gelatin. At 55°C. growth was abundant in 1 per cent glucose gelatin in sixteen hours. After one week the gelatin still solidified in cold water.

Bouillons. At 55°C. there was a slight turbidity in bouillons containing fructose, galactose, glucose, lactose or maltose. There was no growth in plain, glycerol, sucrose, or inulin bouillons.

Potato. No growth occurred on potato.

Carrot. No growth occurred on carrot.

Uschinsky's solution. No growth occurred in Uschinsky's solution.

Dunham's solution. No growth occurred in Dunham's solution.

Litmus milk. When incubated at 55°C. litmus milk was coagulated in from twelve to twenty hours. The litmus was reduced, sometimes before coagulation and sometimes after. A pink band formed at the top which was a little wider than with typical *Streptococcus lactis* cultures. There was no digestion of the milk.

Plain milk. Aside from the color change, the action was the same as in litmus milk.

Biochemical features

Gas formation. No gas was formed in bouillons containing various materials or in milk.

Oxygen relation. The organism was facultative.

Indol. Indol was not formed.

Reaction change. Milk incubated at 55°C. developed acid rapidly. An acidity of from 0.4 to 0.45 per cent (calculated as lactic acid) was reached shortly after coagulation occurred and further incubation failed to increase it. The acid produced in milk was largely lactic and of the *d* type.

Acid[1] was produced in bouillons containing fructose, galactose, glucose, maltose or lactose, but not in plain, glycerol, sucrose or inulin bouillons.

Temperature relationship. Growth was rapid at 55° and 65°C. At 45°C. it was questionable and at 37°C. or lower it did not occur. At 75°C. it was somewhat slower than at 55° or 65°C.

[1] Determined by the addition of brom-thymol-blue.

SUMMARY

In one of the plants in the state, skim milk intended for drying was pasteurized at from 71° to 77°C. and then held hot in a wooden tank for a considerable period, sometimes up to twenty-four hours. Occasionally the milk coagulated and, since the temperature fell slowly, this occurred when the milk was still quite hot.

Two samples of normal skim milk from the plant were studied in the laboratory. A strict thermophile which rapidly coagulated milk at 71°C. was isolated. The coagulation was due to the development of acid; lactic acid of the *d* type was produced while the volatile acid formation was very low. The organism only slightly increased the soluble nitrogen in milk in which it was grown. It is believed to be an undescribed species and the name *Bacillus calidolactis* is proposed for it.

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CHEMICAL STERILIZATION OF MILK BOTTLES IN RELATION TO TUBERCLE BACILLI

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This investigation was undertaken to determine the efficiency of certain chemicals in sterilizing milk bottles infected with tubercle bacilli.

The problem under consideration presented itself in the study of the larger problem of securing for sanatorium patients, inmates of State Institutions, etc., thoroughly safe milk, the ingredients of which should be present in the proper relative quantities in each instance. In order to insure an equable distribution of cream and at the same time to leave no possibility of serving unsafe milk to any individual in sanatoria and other institutions, the State Board of Health has recommended serving bottled pasteurized milk in the original bottle. Where this method has been adopted the institutional manager has, as a rule, instituted the practice of serving straws with each bottle of milk, thereby doing away with the necessity of using a tumbler. It is obvious that by serving the milk directly to tuberculous patients in the bottles, there is an opportunity for the bottles to become infected with tubercle bacilli. It is recognized that an efficient mechanical washing process should reduce the chances of tubercle bacilli remaining in the bottles, but cannot be relied upon to remove all such organisms, and that some subsequent treatment must be applied.

While it had been generally accepted that calcium hypochlorite added to the rinse water reduced the ordinary bacterial count to an insignificant figure, it seemed unreasonable to suppose that the hypochlorite *per se*, of the available chlorine strength usually

recommended, could kill tubercle bacilli should they be present in the milk bottle. It therefore seemed desirable to conduct certain experiments to determine the efficiency of calcium hypochlorite solution for this purpose, and if it were found to be inefficient, to study the results obtained with certain other chemicals.

Three series of experiments are recorded. For each series a fresh sputum specimen was obtained from an open case of tuberculosis in a sanatorium. Each specimen was rendered thoroughly homogeneous by shaking in an electrical shaking machine. All experiments were performed at room temperature.

SERIES I

The first series of experiments was begun and animals inoculated on June 21, 1926, the sputum having been obtained on the previous day. Microscopic examination of the specimen showed from 7 to 12 tubercle bacilli in every microscopic field. Two guinea pigs each inoculated with 0.25 cc. sputum, showed marked lesions of tuberculosis at site of inoculation, in inguinal glands and spleen, on autopsy three weeks after inoculation.

One-half cubic centimeter of the sputum was placed by means of a pipette on the bottom of each of thirteen clean pint milk bottles. To each of four bottles was added 250 cc. of calcium hypochlorite solution containing 10 parts per million available chlorine. The contents of each bottle were agitated by shaking and the bottles were allowed to stand one-half minute, one minute, two and five minutes, respectively. At the end of the time limit sufficient sodium thiosulphate solution was added to completely neutralize the available chlorine, the ortho-tolidin test being used to show that no available chlorine was present after neutralization. To each of four other bottles was added 250 cc. of calcium hypochlorite solution containing 20 parts per million available chlorine and to each of a third set of four bottles was added 250 cc. of hypochlorite solution containing 30 parts per million available chlorine. The same time limits were used as in the first dilution and neutralization accomplished in the same way.

As a control 250 cc. calcium hypochlorite solution containing 30 p. p. m. available chlorine was neutralized with sodium thiosulphate, tested with orthotolidin, and then added to the thirteenth bottle containing 0.5 cc. of sputum.

The contents of the bottles were then centrifugalized at high speed, 2500 revolutions per minute for fifteen minutes, and 2 guinea pigs were inoculated with sediment from each bottle. One pig from each pair was chloroformed and autopsied on the seventeenth day after inoculation; its mate was autopsied on the twenty-third day after inoculation. Every pig showed extensive

TABLE 1

Showing results of treatment of sputum containing tubercle bacilli with varying strengths of calcium hypochlorite

TIME OF EXPOSURE	AVAILABLE CHLORINE		
	10 p.p.m.	20 p.p.m.	30 p.p.m.
minutes			
½	+	+	+
1	+	+	+
2	+	+	+
5	+	+	+

+ indicates that guinea pigs developed tuberculosis.

lesions of tuberculosis with abscesses at inoculation sites, enlargement of inguinal glands and involvement of spleen, and some showed in addition, tubercles in the liver and mesentery.

SERIES II

A similar set of experiments was then made using chloramine[1] in dilutions of approximately 10, 30, 50, and 100 parts per million available chlorine in place of calcium hypochlorite. For this series the sputum was obtained on November 25 and the tests made on November 26. Microscopic preparations of the sputum after shaking showed from 4 to 6 tubercle bacilli per field.

The chloramine solutions were retitrated at the time of use and

[1] Chloramine used in these experiments was prepared by the action of ammonium chloride on calcium hypochlorite in solution.

actual readings were as follows: 7, 28.5, 53 and 95 parts per million available chlorine. The time periods of exposure to each mixture were one, three and five minutes. Two guinea pigs were inoculated in each test. Control tests, using two pigs for each, were made with sputum treated with each dilution of the neutralized chloramine solution; with sputum treated with thiosulphate solution in an amount equivalent to the largest amount used in any of the above tests, to which 250 cc. saline had been added; and with the untreated sputum. All control pigs showed lesions of tuberculosis when autopsied thirty days after inoculation. The test pigs showing abscesses at inoculation site

TABLE 2

Showing results of treatment of sputum containing tubercle bacilli with varying strengths of chloramine

TIME OF EXPOSURE	AVAILABLE CHLORINE			
	7 p.p.m.	28.5 p.p.m.	53 p.p.m.	95 p.p.m.
minutes				
1	+	+	+	+
3	+	+	+	−
5	+	−	+	−

+ indicate guinea pigs developed tuberculosis; −, guinea pigs remained normal.

and enlargement of inguinal glands were autopsied on the thirtieth day after inoculation. All showed at autopsy tubercles in spleen and some tubercles also in liver and mesentery. The remaining pigs were autopsied on the fiftieth day after inoculation and showed all organs normal and an average net increase in weight of 310 grams.

The centrifugalization of these specimens was done in 250 cc. cups and some difficulty was encountered in keeping all the sediment down as it was somewhat flaky. This probably accounts for the negative findings on the pigs inoculated with the solution containing 28.5 parts per million available chlorine for five minutes' exposure. From this series of tests it appeared that the lowest strength of chloramine effective under these conditions was 95 parts per million for a period of three minutes.

SERIES III

On January 28, 1927, a third series of tests was made, the sputum having been obtained on January 27. Each field of a microscopic preparation showed from 4 to 6 tubercle bacilli. A few minor changes in technic were adopted. Instead of pint bottles, half pint bottles were used so that the disinfecting solution tested would entirely fill the bottle, leaving no opportunity for the sides of the bottle above the solution to remain unaffected by the solution. In neutralizing the solutions, agitation was accomplished by stirring with sterile glass rods instead of by shaking the bottles as in the earlier series of tests. The solutions containing the treated sputum were first centrifugalized in 250

TABLE 3

TIME OF EXPOSURE	CALCIUM HYPOCHLORITE AVAILABLE CHLORINE					CHLORAMINE AVAILABLE CHLORINE			
	49.6 p.p.m.	106 p.p.m.	200 p.p.m.	300 p.p.m.	500 p.p.m.	93 p.p.m.	177 p.p.m.	219 p.p.m.	396 p.p.m.
minutes									
½				+	+			−	−
1	+	+	+	+		+	−	−	
3	+	+				−			

+, indicate guinea pigs developed tuberculosis; −, guinea pigs remained normal.

cc. cups, the supernatant fluid decanted to about 15 cc., and the lower strata again centrifugalized in 15 cc. tubes. The sediment in each instance was used to inoculate two guinea pigs.

It was intended to use calcium hypochlorite in dilutions of approximately 50 parts per million available chlorine for one and three minutes, and both calcium hypochlorite and chloramine in dilutions of 100 p.p.m. available chlorine for one and three minutes, 200 p.p.m. for one minute, 300 p.p.m. one-half and one minute, and 500 p.p.m. for one-half minute.

The solutions were prepared and titrated about four hours previous to using them for the tests, and immediately after completion of the tests the solutions were re-titrated. It was found

that the chloramine solutions had already deteriorated somewhat.[2] The actual strengths on re-titration are indicated in Table III, which gives results obtained.

Control tests using but one guinea pig for each were made on the sputum and on the sputum treated with the neutralized solutions of calcium hypochlorite and chloramine as in series II. All control pigs showed lesions of tuberculosis when autopsied thirty-one days after inoculation. Such of the test pigs as showed palpable lesions at the end of four weeks were chloroformed and autopsied twenty-eight to thirty-one days following inoculations. The remaining pigs were chloroformed and autopsied at the end of two months when no evidence of tuberculosis was found. All of the negative pigs showed an average increase in weight of approximately 210 grams after inoculation.

This series of experiments checks very closely with series II, in that a strength, of chloramine of approximately 100 parts per million available chlorine for three minutes is shown to be effective in killing tubercle bacilli even when present in large numbers. In this series, as in series I, all dilutions of calcium hypochlorite were unsuccessful in destroying tubercle bacilli.

CONCLUSIONS

From these experiments it appears that calcium hypochlorite solution containing available chlorine in dilutions usually advised for disinfecting milk apparatus and utensils in milk plants, and even up to 500 parts per million available chlorine can not be depended upon for the destruction of human tubercle bacilli. It further appears that the use of chloramine in a strength of 93 to 95 parts per million available chlorine for a period of three minutes is effective.

The results further indicate that stronger solutions of chloramine applied for a shorter time are also effective.

The results of this investigation should not be interpreted to apply to substances or conditions other than those included in this study.

[2] Chloramine in weak solutions deteriorates rapidly and should be kept in a strong solution securely stoppered in an amber-colored bottle and diluted to the desired strength when needed for immediate use.

THE INSOLATION OF CERTAIN CULTURE MEDIA

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The profound influence of sunlight on the life of the earth is well recognized, the lower forms as well as the higher being affected by it, but not necessarily in the same manner. Many species of bacteria may be destroyed or their growth inhibited by the action of sunlight or, artificially, by ultraviolet rays while on the contrary certain deficiency diseases in higher organisms are ameliorated by treatment directly with similar light or indirectly by treatment with irradiated materials. It is reasonable to expect that chemical compounds of an organic nature will also be affected by such light. Raoult (1871) found that cane sugar in solution was partially changed to glucose by the action of sunlight. In experimenting with anthrax spores Roux (1887) found that media exposed to sunlight were made so toxic after four hours insolation that spores would not germinate on them. He attributed this condition to the oxidation of the carbohydrate of the medium. On the other hand Gladstone and Tribe (1883) question Raoult's results and conclude, "that neither light nor air, singly or jointly, changed cane sugar to glucose in aqueous solution at ordinary temperatures." It is quite possible that much of this earlier work was done with unsterile solutions and that the changes observed may have been due more to biological than to solar action. More recent work includes that of Spoehr (1912) who exposed some of the fatty acids to sunlight for a year with the result that all except formic acid decomposed into formaldehyde and carbon dioxide; Neuberg and Peterson (1914) produced alkali from neutral salts of organic acids by subjection to sunlight. Bovie (1916) who reviewed the literature on the

subject of the effect of light on living organisms, concluded that these effects were due to the formation of toxic substances. While Coblentz and Fulton (1924) reported that Browning and Russ detected no effect on agar by exposure to ultraviolet light yet from their own experiments, they came to an opposite conclusion, especially with reference to high radiation intensities. Woodrow, Bailey and Fulmer (1927) found that toxic substances were formed in yeast media due to irradiation with ultraviolet light and that the toxicity increased with the length of exposure. This toxicity was found not to be due to changes in hydrogen ion concentration of the media.

In the preparation of culture media in the laboratory it is often inadvertently exposed to sunlight for relatively short periods of time. When such is the case the question sometimes arises as to whether this exposure has caused the formation of substances either detrimental or stimulative to the growth of the organisms for which the media were prepared.

In the following work an endeavor has been made to estimate the effects produced through insolation of media by using the growth of organisms and alteration in hydrogen ion concentrations as indices. The ultraviolet rays in the sunlight are, of course, largely filtered out by the glass containers of the media but as it would be rather an extraordinary procedure to store media in quartz containers, this work does not consider the action of sunlight under such conditions.

METHODS AND RESULTS

Four kinds of media were used in this experiment; beef broth and agar[1] and soil extract broth and agar.[2] Sixty-milliliter portions

[1] The formula used for the broth was as follows:

Liebig's beef extract	5 grams
Sodium chloride	3 grams
Peptone (Difco)	10 grams
Distilled water	1000 ml.

and the agar was the same except for the addition of 10 to 12 grams of agar (Bacto). The pH of each was adjusted to 6.8.

[2] These media were made according to the methods described by F. Löhnis (1913).

of each of these were placed in 300 ml. Erlenmeyer Pyrex glass flasks and these were plugged with rubber stoppers, each stopper carrying a glass tube about 3 inches long and $\frac{1}{8}$ inch in diameter, plugged with cotton. After sterilization the tubes were sealed to prevent evaporation of the media. Half of each lot were placed in a dark closet and the other half exposed to sunlight for a total of two hundred seventy hours. Determinations of pH were made at intervals by the colorimetric method described by Clark (1922) and the results are given in table 1. Each kind of media was tested as to its efficiency as a bacterial food after ten and two hundred seventy hours exposure to sunlight, controls being maintained by means of the flasks kept in the dark. The following bacteriological method was used: flasks of the sun-exposed media and control flasks were inoculated with similar units of suitable organisms. In the case of the beef media, *B. subtilis* and an organism from the leaf tips of *Dioscorea macroura* were used and with the soil extract media various strains of nodule-forming organisms. After a two-day incubation for the beef media and five days for the soil extract media the bacterial growth was homogenized with the media plus water by machine shaking and three plates made on appropriate agar from each of three similarly treated flasks. Following incubation, counts were made of the colonies that developed and the average numbers of bacteria in 1 ml. of media calculated. These results are given in table 2.

Differences in growth of the organisms after two hundred seventy-hour incubations were noted. *B. radicicola* developed as a raised, slimy growth on the unexposed soil extract agar,

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Air dry soil	500 grams
Tap water	1000 ml.

These were heated, after thorough mixing, at 15 pounds pressure for fifteen minutes. After stirring and filtering the filtrate was made up to 1,000 ml. with tap water, and to this were added

Dipotassium phosphate	0.5 gram
Mannito	10.0 grams

and in the case of the Agar 10-12 grams of agar (Bacto). The pH of each was adjusted to 6.8.

TABLE 1

pH determinations on media tested

MEDIA TESTED	HOURS OF EXPOSURE																	
	0		10		28		51		75		103		169		213		270	
	Control	Sun	Control	Sun	Control	Sun	Control	Sun	Control	Sun	Control	Sun	Control	Sun	Control	Sun	Control	Sun
Beef agar	6.5								6.5	6.1	6.5	5.9	6.5	5.5			6.4	5.5
Beef broth (1)*	6.6		6.9	6.7	6.7	6.4	6.5	6.0	6.1‡	5.5‡					6.7	6.0	6.9	6.1
Beef broth (2)†	6.7		6.8	6.7														
Soil agar	6.7								6.3	5.6	6.4	5.5	6.1	5.2			5.5	5.2
Soil broth (1)*	6.5		6.9	6.9	6.6	6.7	6.6	6.6	6.1‡	6.1‡					6.8	6.8	6.7	6.8
Soil broth (2)†	6.7		6.6	6.5														

* Made November 23, 1925. † Made November 29, 1926. ‡ Low results are due to indicator error.

TABLE 2

Numbers of bacteria developing on media exposed and unexposed to sunlight

Expressed as millions of colonies

MEDIUM	DIOSCOREA MACROURA ORGANISM			B. SUBTILIS			436*			372*			239*			240*		
	Control	10 hours exposure	270 hours exposure	Control	10 hours exposure	270 hours exposure	Control	10 hours exposure	270 hours exposure	Control	10 hours exposure	270 hours exposure	Control	10 hours exposure	270 hours exposure	Control	10 hours exposure	270 hours exposure
Beef agar				6.4		1.3												
Beef broth (1)*	761	1,188																
	765		1,262	2.3		3.0												
Beef broth (2)	620	638		2.2	16.3													
Soil extract agar							566		450	133		285	213		78			
Soil extract broth (1)							80	65								62	112	
							112		134							148		157
Soil extract broth (2)							352	310								398	356	

* 436, 372, 239, and 240 are the culture numbers of alfalfa, clover, vetch and soybean strains of legume bacteria respectively.

while the growth on the agar exposed to the sun was thin and watery. In the case of the soil extract broth the growth was quite flocculent in character in that exposed to sunlight while the unexposed broth developed a uniform suspension of bacteria. The *Dioscorea macroura* organisms on beef broth showed the same type of growth in exposed and unexposed flasks while in the case of *B. subtilis* there was a thin film on the surface of the liquid in the sunlight exposed flasks and a heavy white scum on the others. When the media were exposed to ten-hour periods of sunlight, no differences of growth were noted between the irra-

TABLE 3

Numbers of bacteria developing on media stored in the dark for one year and then exposed to sunlight

MEDIUM	DIOSCOREA MACROURA ORGANISM		436*		240*	
	0 hours	10 hours	0 hours	10 hours	0 hours	10 hours
Beef broth	761†	1,188				
Soil extract broth			80	65	62	112

* 436 and 240 are the culture numbers of alfalfa and soybean strains of legume bacteria respectively.

† Organisms given in millions.

diated media and the controls. The long exposures to sunlight resulted in a bleaching action on the media.

A test was also made on flasks of beef and soil extract broth that had been kept in the dark closet for over a year. Half of these were exposed to sunlight for ten hours and the same experimental procedure as before was followed, the bacteriological results being shown in table 3. There were no changes in the pH readings due to the exposure to sunlight.

SUMMARY

Culture media of four kinds have been exposed to the rays of the sun for various periods of time and tested for changes in hydrogen-ion concentration and efficiency in supporting the growth of certain bacteria.

In the case of beef agar and broth and soil agar changes in hydrogen-ion concentration were noted after two hundred seventy-hour exposures, while changes in the other media were not found.

There were, in some cases of two hundred seventy-hour exposures, marked differences of appearance between the bacterial growth on the media insolated and on that unexposed and colony development on these media for both periods of time indicate variations but they are not sufficiently consistent to warrant definite conclusions.

The significant conclusion from this work is that appreciable changes to which the organisms used are sensitive, in these culture media occur only, if at all, after an exposure to sunlight which is much greater than any which would be inadvertently given.

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A STRAIN OF CLOSTRIDIUM WELCHII CAUSING ABNORMAL GASSY FERMENTATIONS IN EMMENTHAL OR SWISS CHEESE

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A frequent difficulty encountered in the manufacture of Emmenthal or Swiss cheese is an abnormal gassy fermentation. These gassy cheeses are known to the maker as "niszler" or "pressler" cheeses, depending upon the rapidity and extent of the fermentation. Although much work has been done on this problem both here and abroad, and in spite of the now general practice of employing a starter of a high-acid-producing organism to combat abnormal fermentations, a large number of niszler and pressler cheeses result each season. In some factories the trouble is quite serious, an appreciable number of the cheeses being gassy, while in other factories it amounts to but a few cheeses in a month.

A "niszler" cheese is characterized by a uniform distribution of very small holes throughout the cheese. Frequently this condition is not detected until the cheese is several weeks old. A "pressler" cheese is one in which the gassy fermentation is in evidence on the press. The gas holes are usually larger than those found in the niszler cheese and are in most cases confined to an area near the surface of the cheese. When the fermentation is particularly vigorous the cheese "huffs" and in extreme cases portions of it are forced between the board and the hoop, forming large lips.

Those who have studied the gassy fermentations of the cooked cheeses are in accord as to the chief cause of the trouble, namely, the colon-aerogenes group of bacteria. Orla-Jensen (1921), Barthel (1910), and others have shown that the butyric acid

bacilli may also be responsible for gassy fermentations in cheese. An investigation as to the cause of an unusually large number of niszler and pressler cheese in one factory led to the isolation of a strain of *Clostridium welchii* from one of the defective cheeses. This organism was found to be responsible for the abnormal fermentation.

Repeated efforts to isolate a gas-forming aerobic organism from the defective cheeses at this factory were unsuccessful. An attempt was then made to isolate a spore-forming anaerobe. Portions of the defective cheese were ground in sterile sand. Eight-ounce bottles containing 200 cc. of plain infusion broth, pH 7.0, were boiled for thirty minutes, quickly cooled, and inoculated with generous portions of the sand-cheese mixture. These were incubated for three days at 37°C. Tubes containing about 15 cc. of melted plain infusion agar were inoculated with about 1 cc. of the broth and then heated to 75°C. for ten minutes, cooled, and incubated at 37°C. After twelve hours' incubation the agar in the tubes was blown with gas. Broth cultures made from the tubes showing gas were plated on plain infusion agar and the plates were incubated for three days at 30°C. in a sealed desiccator in which the air had been replaced with hydrogen. Copper gauze pockets containing palladianized asbestos were suspended inside the desiccator to take care of the oxygen. Colonies were picked from these plates into plain freshly heated infusion broth, sealed with vaseline, and incubated for twenty-four hours at 37°C. Those tubes which showed gas were again plated. Four platings were made to insure the purity of the culture. The cultures obtained from the fourth plating appeared to be pure when examined microscopically.

The organism thus isolated was a thick, non-motile, Gram-positive bacillus, occurring singly, with a few short chains. It produced a stormy fermentation in milk. In chopped meat medium a dense uniform turbidity with evolution of gas appeared within twenty-four hours. The turbidity eventually settled out leaving a clear supernatant fluid. The meat was apparently unchanged and gave no evidence of blackening after a month's incubation.

Glucose, galactose, sucrose, maltose, inulin and starch were fermented with evolution of gas; while raffinose, mannitol, glycerol and salicin were not. The fact that all the cultures fermented inulin but not glycerol would identify the organism as belonging to group 3 in the classification of Simonds (1915). This strain is non-pathogenic.

A large number of cheeses have been made from milk inoculated with a milk culture of this strain of *Clostridium welchii*. The use of large amounts of a pure milk culture of *Lactobacillus bulgaricus* together with an increased cooking temperature had little or no effect upon the development of the organism in cheese. This method is efficacious, however, in suppressing the growth in cheese of members of the colon-aerogenes group.

It has been a not uncommon belief among cheesemakers that "niszler" cheese was caused by an organism different from that which produced gassy cheese of the "pressler" type. Experimental cheeses displaying both of these types of gassy fermentation have been produced by this organism. On a few occasions the cheeses made from milk inoculated with this strain of *Clostridium welchii* showed little or no gas, although there was no known variation in the method of manufacture. It appears from these experiments that some factor or group of factors, as yet undetermined, plays an important part in the occurrence of gassy fermentations in cheese, especially those occurring in the cooked cheeses. The results of a study of these factors will be presented in a later paper.

SUMMARY

1. A non-pathogenic strain of *Clostridium welchii* was isolated from a Swiss cheese which showed an abnormal gassy fermentation.

2. Experimental cheeses made from milk inoculated with this organism showed both "niszler" and "pressler" types of gassy fermentation.

3. It is evident that some factors or group of factors other than the presence of the gas-producing organism and the present method of manufacture play an important part in determining the type of gassy fermentation in cheese.

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IS THE STATISTICAL METHOD APPLICABLE TO THE BACTERIAL PLATE COUNT?

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Statistical analyses of biological data are quite common today. The method is applied to various types of data, oftentimes distinctly to the detriment of well established practices which are supported by opinions of capable men. The recent criticisms of the Koch plate method for the enumeration of bacteria in milk is a case in point. Much good has come from the use of the agar plate in the past. Through its agency as a control measure, our milk supplies as a whole are immeasurably better now than ever before. That there are faults in the method cannot be denied; duplicate plates often vary to a large degree. Variations in plate counts are obtained by different workers with the same milk. The explanation is not far to seek when one studies the report of the Referee in which the needless variation of a supposed "Standard Procedure" is shown to exist (Breed, 1926).

The criticisms that the statistician makes are of a much more serious nature. Wright and Thornton (1927), using the same medium, the same methods, by the same operator, report that a "variation of less than 100 per cent within a series cannot be depended upon . . . . the extreme variation was 1500 per cent. The average coefficient of variability for all counts was 25 per cent. High and low extremes were 57 and 15 per cent." If all of this is generally true then the final statement in their brief report is likewise true when they say that "this method is no more accurate for grading milk than other means available." It must be borne in mind however that with the agar plate as a foundation there has been built up a very stable system of milk control. Destroy confidence in the agar plate and milk control will suffer.

The statistical method in itself as a mathematical procedure is free from criticism but there are many pitfalls for the unwary in the unrestricted use of the method. E. B. Wilson (1923) has pointed out that "a method is a dangerous thing unless its underlying philosophy is understood and none more dangerous than the statistical. Our aim should be with care to avoid, in the main, erroneous conclusions. In the long run it is only clear thinking . . . . that wins the strong hold of science."

The results reported by Wright and Thornton were so pointed that we decided to determine for our own working conditions the probable error of the plate method. We plated nine samples of milk in a single series using 75 plates to a series. In this work the procedure in every detail followed the accepted standard methods. The dilutions were chosen after a direct count had been made upon the milk, and were so made that the dilution used was one of 100 cc. From this dilution bottle 75 plates were made which were previously marked so that the first cubic centimeter of the dilution was placed in the plate marked one, the second in the plate marked two, and so on to the end. One pipette was used for 10 plates and then discarded. In this work only calibrated pipettes were used to avoid error from this source. Twelve-cubic-centimeter amounts of agar were kept in tubes at a constant temperature of 40°C. and this was poured into the plates, twenty plates being poured at a time as the experiment progressed. The plates were stacked in the incubator in piles of six, with spaces of one or two inches between the stacks. They were incubated for forty-eight hours at 37°C. after which time they were counted. Throughout the experiment the same person performed all the operations. The incubator is a room 10 feet on a side in which a fan was kept in motion.

The data so obtained are summarized in table 1 in which are given the low, high and mean counts for each of the nine trials. The calculated standard deviation and coefficient of variability are also given.

It is quite apparent that in the first five trials the results indicate that from a statistical point of view the method is of little value. The spread from low to high in each case is very large.

It was apparent however in studying the tabulated data from which the summaries of table 1 were taken that the high counts usually came from the plates made at the end of the experiment. This was so apparent that a graph was made in which the bacterial counts were expressed as ordinates and the number of plates as abscissae. It will be recalled that when the plates were made the precaution was taken to number the plates in the order in which they were used, thus giving a measure of time. When results of each of the experiments were plotted there were disclosed some interesting facts. Since trial no. 1, however, is quite typical

TABLE 1

High and low bacteria counts with statistical constants for nine milk samples

TRIAL	LOW	HIGH	MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIABILITY
					per cent
1	38,000	1,600,000	292,506 ±30,531	392,000 ±21,607	134.0
2	58,000	490,000	183,693 ± 7,406	95,100 ± 5,240	51.4
3	3,000	60,000	13,773 ± 661	8,490 ± 467	61.6
4	5,000	110,000	26,653 ± 2,000	25,700 ± 1,416	86.4
5	48,000	620,000	138,333 ± 8,956	115,600 ± 6,370	83.7
6	53,000	148,000	108,640 ± 1,456	18,700 ± 1,030	17.0
7	37,000	63,000	46,346 ± 487	6,260 ± 344	13.5
8	6,000	66,000	21,013 ± 804	10,330 ± 569	49.0
9	6,000	118,000	34,013 ± 1,853	23,800 ± 1,311	69.9

it may be discussed at length since the other four of the first five are all similar and differ from it but slightly. In making the graphs the scale of the ordinates has been changed in each case to fit the spread of the counts for the experiment. It will be seen from figure 1 that while the counts vary but little for the first 40 plates poured there is a sharp rise in the count at this point. The mean of the 75 is 292,000 and is indicated in the figure, but it lies well above the range of the count of the first 40 plates whose mean is shown as 62,000. There are a few counts near the end of the trial which are also low but the majority after the first 40 steadily rise to a high point of 1,600,000. This increase in number of bacteria in the plates made toward the end of the experiment

cannot reasonably be considered as due to the growth of the organism since the temperature of the milk was about 50°C. and that of the dilution water about 20°C., while the average time necessary to make the 75 plates was less than 90 minutes. It is also unlikely that the increase in the number of organisms in the plates could be due to carelessness since it would have taken from

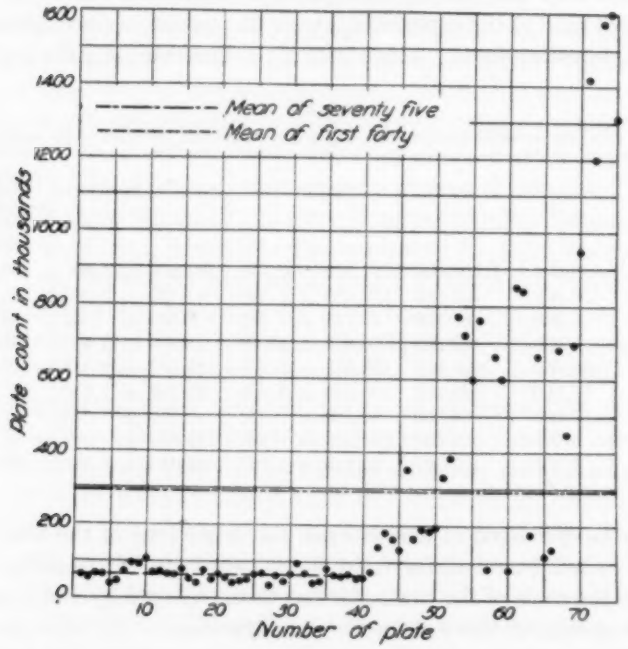

FIG. 1. DISTRIBUTION OF BACTERIA COUNTS THROUGH 75 PLATES OF TRIAL I, SHOWING MEAN OF THE 75 PLATES AND MEAN OF THE FIRST 40

20 to 40 cc. of the dilution to obtain the high counts recorded. Obviously a coefficient of variability of 134 per cent indicates that the mean value of all the counts in experiment 1 is meaningless. The mean of the first 40 plates for all of the trials was next calculated.

TABLE 2

Statistical constants for 75 plates compared with those of the first 40 plates

TRIAL	MEAN		STANDARD DEVIATION		COEFFICIENT OF VARIABILITY	
	75	First 40	75	First 40	75	First 40
					per cent	*per cent*
1	292,506 ±30,531	62,970 ±1,739	392,000 ±21,607	16,300 ±1,229	134.0	25.8
2	183,693 ± 7,406	134,400 ±4,909	95,100 ± 5,240	46,000 ±3,470	51.4	34.0
3	13,773 ± 661	11,625 ± 368	8,490 ± 467	3,450 ± 260	61.6	29.6
4	26,653 ± 2,000	15,150 ± 673	25,700 ± 1,416	6,310 ± 481	86.4	41.6
5	138,333 ± 8,956	83,100 ±1,430	115,600 ± 6,370	13,400 ±1,010	83.7	16.1
6	108,640 ± 1,456	103,850 ±2,113	18,700 ± 1,030	19,800 ±1,493	17.0	19.0
7	46,346 ± 487	44,255 ± 515	6,260 ± 344	4,830 ± 364	13.5	10.0
8	21,013 ± 804	19,050 ±1,000	10,330 ± 569	9,380 ± 707	49.0	49.2
9	34,013 ± 1,853	17,375 ±1,163	23,800 ± 1,311	10,900 ± 822	69.9	62.7

In table 2 the means for both the 75 plates and the first 40 plates are given together with the statistical constants. It will be seen that the coefficient of variability for trial no. 1 decreases from 134 per cent for the 75 plates to 25 per cent for the first 40 plates. The plates used in a routine analysis would be the first two and very little variation is found.

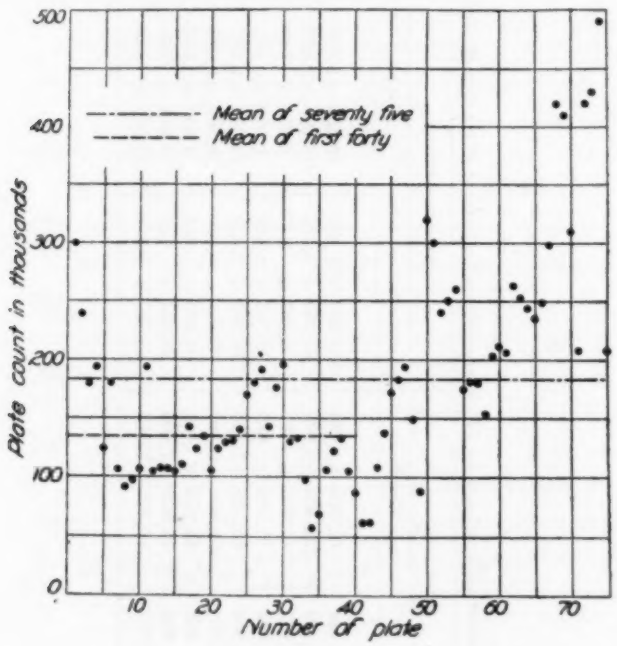

FIG. 2

Wright and Thornton report a coefficient of variations of 57 per cent. It is our suggestion that these authors failed to take into consideration the time factor which is evidently of importance. Had we not considered this in our work and had we counted plate number one first, plate 75 second, plate 2 third, and so on, we would have found the same coefficient of variability we did

find, 134 per cent, but there would have been no explanation for it with a resulting misinterpretation of our findings. The reason for the increase in plate counts in the plates numbered above 40 is not known. It was thought that the clumps broke up on standing, but a microscopical study of the dilutions did not substantiate this opinion, although it seems to be the only

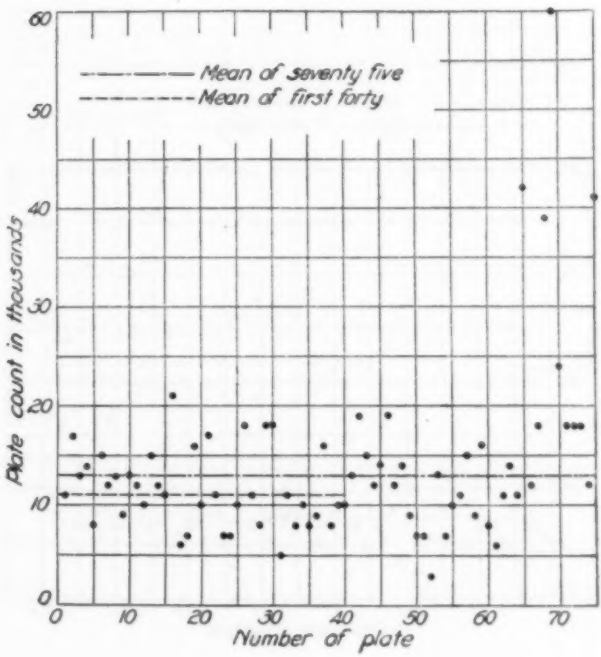

FIG. 3

rational explanation. It is also suggested that clumping is found at the normal pH of the milk which is offset by the addition of the milk to the water and, although the microscope does not disclose the fact, the clumps may be less firmly held together with the change in pH. We are at present working upon an explanation which will be reported in the future.

Figures 2 to 5, which are obtained from trials 2 to 5, show much the same increase in counts in the higher number plates. The decreases in the statistical constants are shown in table 2.

Figures 6 to 8 show what might be considered normal distribution of counts. Figure 6 is interesting since it shows a slight but progressive increase in counts, although the coefficient of varia-

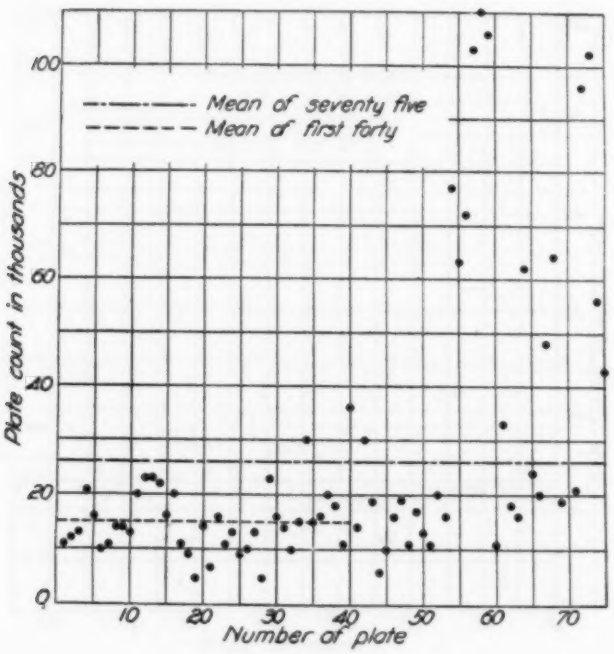

FIG. 4

bility is low. Further mention of this trial will be made later in this paper.

Trial 9 is of more than passing interest. A low count of 6000 and a high count of 118,000 are recorded. Plotted as a scatter diagram in figure 9 it will be seen that there are two distinct groups of counts; one from the plates numbered below 40 and the

other from the plates numbered above 40. The counts are higher after the first 40 plates but the increase is not a gradual one as in the first five trials. The mean for the 75 is 34,000, that for the first 40, 17,000, but the coefficients of variability are the same; indicating that they both have the same relative magnitude of error. This is doubtless true mathematically, but from the

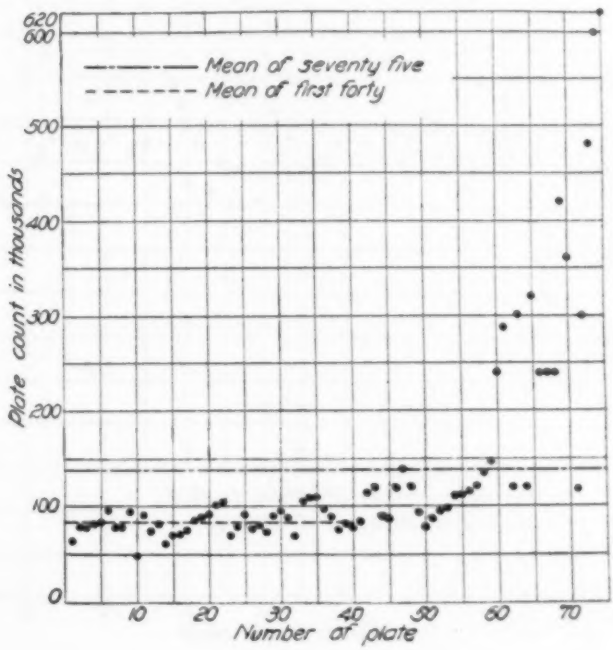

FIG. 5

standpoint of the milk control a range of 6000 to 37,000, high though it may be, is more to be desired than the far greater one of 6000 to 118,000.

The foregoing would resolve itself into the question: Can we use the coefficient of variability or attempt a measure of relative variability as seems to be the increasing practice? Pearl (1927)

says that particular constant has never been an entirely satisfactory one for biologists to use. We think that Pearl is right. In support of this statement let us assume two hypothetical cases which incidentally are not far removed from a possibility in milk

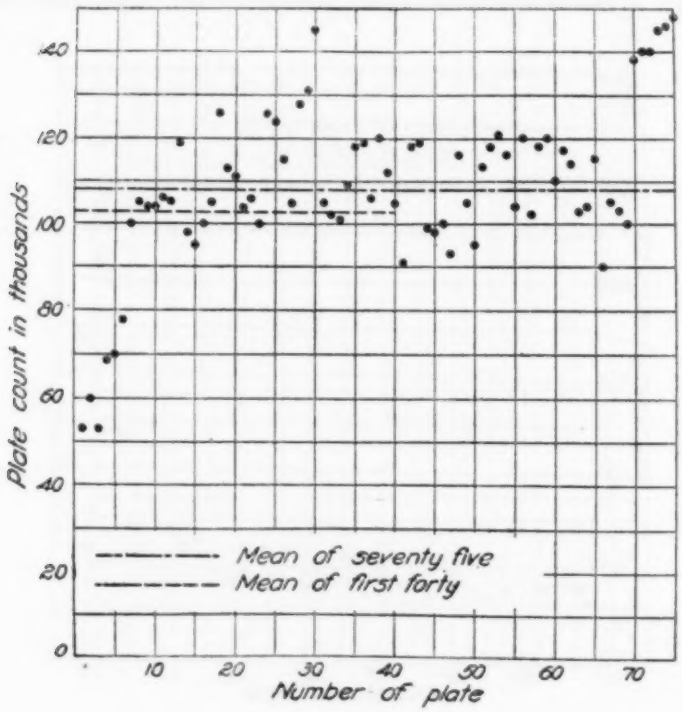

FIG. 6

control. Assume that we have two sets of data; as in table 3 (*a*) with nine scores of 10 and one of 100, and (*b*) nine scores of 100 and one of 10.

The mean for (a) is 19, for (b) 91. The standard deviation would be the same in each case. The coefficient of variability of (a) is 144 per cent, while that of (b) is but 29 per cent, but *the*

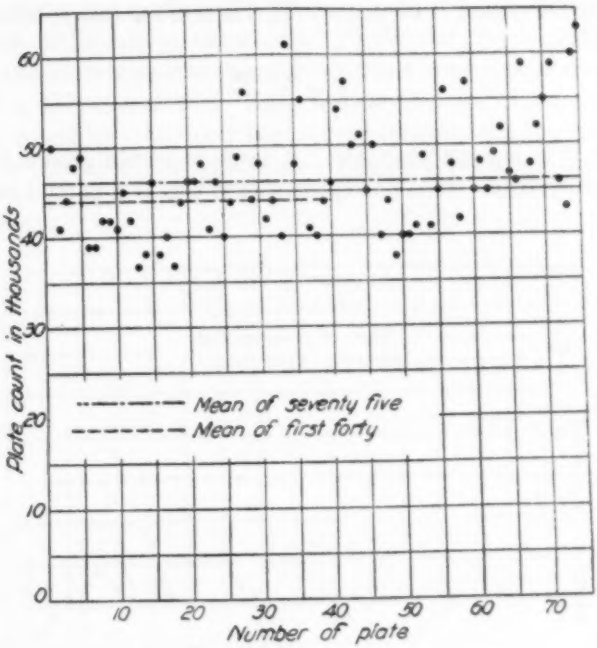

FIG. 7

TABLE 3

	(a)	(b)
	100	10
	10	100
	10	100
	10	100
	10	100
	10	100
	10	100
	10	100
	10	100
	10	100
Mean	19	91
Standard deviation	27	27
Coefficient of variability	144.4%	29.7%

standard deviation is the same. The reason for this is that in the formula

$$V = \frac{\sigma \times 100}{m}$$

the m is in the demoninator. A large m would give a small V and conversely. It is our opinion that this is a partial explana-

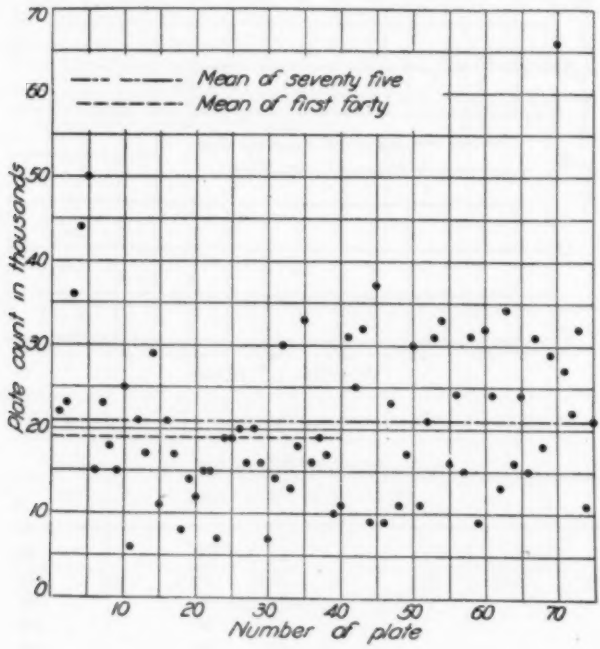

FIG. 8

tion of the low coefficient of variability for trial 6 as compared to the high value of 49 per cent for the same constant for trial 8.

This paper is frankly critical; its object is to point out constructively consequences of too close adherence to a mathematical formula. It would seem well then to mention two recent publica-

tions in which the coefficient of variability has been determined in studies upon the agar plate method. Norton and Seymour (1926) studied the variations resulting from plating a number of samples upon five different media. A statistical analysis of the results was attempted by tabulating the average total counts, the average deviation from the average counts, standard deviation and the

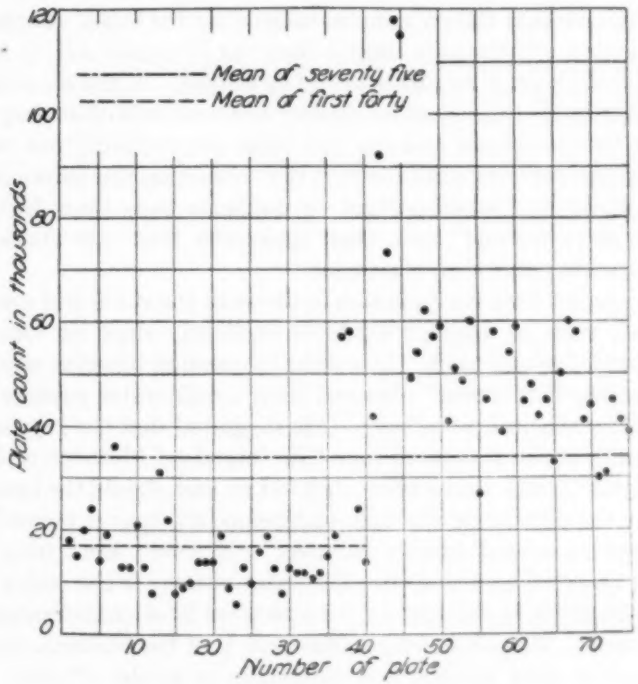

FIG. 9

coefficient of variability. Their average, however, was an average of the variations in the milk samples and has nothing to do with the method. There is no statistical reason for such average being taken and certainly no justification for calculating standard deviations from an average so taken, yet they cite coefficients of variations from 63 to 79 per cent which are abnormally large.

Ellenberger et al. (1927) also have apparently missed the point in a paper comparing the methylene blue reductase test to the agar plate count. They made trials on 79 milk samples. Each sample was divided into six smaller samples and two plates and two tubes were used which were supposed to furnish an opportunity to determine the coefficient of variation for both the agar plate count and the reductase test. It is a question whether they can assume that the six samples taken from the larger sample are all similar. If they are similar then the 12 plates and 12 tubes are hardly enough to constitute a fair sample. If the six samples are not to be considered as similar, and we doubt that they are, then the two plates and the two tubes are certainly not representative; yet they conclude that the "reduction test shows much less variability between check or duplicate tests than does the agar plate method" and they *apparently* have the statistical evidence to prove their statement.

It has not been our intention to discredit the statistical method *per se;* such an attempt would be foolhardy since its value to science is beyond cavil. It is open to question however whether increasing the "scores" obtained from a milk-water mixture is a correct statistical procedure. It is suggested that the key to the difficulty is the statement found in "Standard Methods of Milk Analysis" (1923) to the effect that "in no case should the interval (after the dilution of the milk and before the agar is poured) be allowed to exceed twenty minutes." It would seem from the work reported here that the milk-water mixture is not stable and that dispatch in completing the operation is of considerable importance. When it is fully understood that the bacterial counts cannot be used to split fine differences in grades of milk, but that counts can be used as a splendid means to the end of better milk control, the so-called "errors" will be considered less and less, for after all "probably the best safeguard against error is the certain knowledge on the part of the investigator that error is always possible and seldom absent. Dull the appreciation of this fact by insistance on infallibility of any formula and the way to error is thrown open" (Salmon, 1923).

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THE DECOMPOSITION OF CELLULOSE BY AEROBIC BACTERIA

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HISTORICAL

Kellerman, McBeth and associates (McBeth, 1916) have described a cellulose agar medium consisting of cellulose, precipitated by acids from a cuproammoniacal solution, carefully washed and suspended in a mineral agar medium. Although this medium has been used with success by its authors and some other investigators, it has not been adopted in the work reported here for the following reasons: (1) It is very tedious to prepare; (2) the necessity of using elective cultures before plating rules out the use of the method for enumeration purposes; (3) as the cellulose is completely submerged in these elective cultures, it is impossible for the strictly aerobic bacteria to develop; (4) this inhibition of the aerobic forms is accentuated by the fact that, on plating, the cellulose particles are imbedded in the agar.

Now, it will be shown that many very active cellulose-decomposing bacteria are strictly aerobic and grow only in contact with the air. We can therefore understand why the use of the cellulose-agar method has not revealed the existence of such strict aerobes and why all the species isolated by Kellerman and McBeth are more or less anaerobic.

Bojanowsky (1925), Waksman and Carey (1926) and Winogradsky (1926) suggested the use of a silica gel receiving a mineral solution and some cellulose on the surface, to demonstrate the presence and abundance of cellulose-decomposing bacteria. This method has certainly some value for giving an idea of the relative abundance of these organisms, but the numbers of

organisms developing on the plate do not bear any relation to the number of organisms present in the inoculum.

The object of this article is to present a description of a method that lends itself to the enumeration and isolation of cellulose-decomposing bacteria, as well as a description of some new species.

EXPERIMENTAL

Under aerobic conditions, cellulose is usually readily attacked by many species of filamentous fungi; they rapidly overgrow the cultures and make it difficult to recognize the activities of cellulose-decomposing bacteria. It was therefore important to devise means which would repress the growth of the fungi and favor the growth of the bacteria.

The following experiment reveals that cellulose-decomposing bacteria are greatly favored by a neutral or slightly alkaline reaction; at the same time, the growth of some fungi is retarded under such conditions.

A medium of the following composition was used:

	gm.
$NaNO_3$	1.0
$MgSO_4 \cdot 7H_2O$	0.5
KCl	0.5
$FeSO_4 \cdot 7H_2O$	0.01
H_3PO_4	2.0
Distilled water	1000

The medium was adjusted to different reactions by the addition of KOH. The most alkaline reactions (pH 8.5 to 9.0 to 9.5) are probably of little significance, as phosphate solutions are poorly buffered at these reactions. Twenty cubic centimeter portions of these media were added to Erlenmeyer flasks, with a sheet of paper partly immersed. After sterilization, the flasks were inoculated with a series of cellulose-decomposing organisms obtained from the soil, and incubated at 28°C. The number of days after which cellulose decomposition became apparent, as recognized by disintegration of the paper and microscopic examination, is recorded in table 1.

It was observed also that, when nitrogen salts are used in small concentrations, growth develops more rapidly (table 2).

TABLE 1

Influence of reaction (pH) on the time (days) required for appearance of cellulose decomposition by microörganisms

ORGANISM	REACTION (pH)															
	2.1	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5
Green *Penicillium*			4	3	3	3	3	4	6	6	8	9	9	15	17	
Trichoderma koningi	6	6	3	3	3	3	3	3	5	5	5	6	9	14	16	16
Humicola sp.*			9	3	3	3	3	3	3	3	3	3	3	3	5	5
Actinomyces sp.								9	9	5	5	5	5	5	6	9
Sp. cytophaga										5	4	3	4	5	5	
Orange rod									16	4	4	11	14	14		
Yellow vibrio										4	2	2	2	2		
Colorless vibrio										4	2	2	2	3	4	
Facultative anaerobe W									17	17	8	8	8	14	14	17

* A detailed description of this organism is given by Traaen (Nyt. Magaz. Naturw, Christiania, v. 52, 1914, p. 20).

TABLE 2

*Influence of concentration of $NaNO_3$ on time (days) required for appearance of cellulose decomposition by soil microörganisms**

ORGANISM	CONCENTRATION OF $NaNO_3$				
	0.1	0.5	2.5	5.0	10.0
	grams per liter	*grams per liter*	*grams per liter*	*grams per liter*	*grams per liter*
Sp. cytophaga	3	3	4	4	
Yellow vibrio (Y)	2	2	2	3	3
Colorless vibrio (Co)	4.5	2	4	4.5	
Green vibrio (Gr.)	2	2	2	4	
Slender thread (T)	2	2	2	4	
Facultative anaerobe (W)	8	9	10	13	13
Observations		Optimum		Very poor growth	

* Reaction of the medium pH 7.5

It also seems that in the presence of small amounts of nitrogen salts, *Spirochaeta cytophaga* becomes a little more resistant toward acidity (see table 3).

Due to the method of measurement, these results are of course not absolute, but they serve to indicate that the multiplication of this organism is most rapid between pH 7.0 and pH 7.5, in a medium containing about 0.25 to 0.50 gram of $NaNO_3$ or $(NH_4)_2SO_4$ per liter.

It was attempted to improve this medium by the addition of (*a*) soil extract, (*b*) materials rich in vitamines (aqueous extract of leaves and seeds filtered through Chamberland filter), and (*c*) peptone as a source of nitrogen.

TABLE 3

Influence of combined effects of reaction (pH) and concentrations of $(NH_4)_2SO_4$ or $NaNO_3$ on the time (days) required for appearance of cellulose decomposition by Sp. cytophaga

CONCENTRATION OF $(NH_4)_2SO_4$	pH 5.2	pH 5.7	pH 6.2	pH 6.6	pH 7.1	pH 7.5	pH 8.2
grams per liter	*days*	*days*	*days*	*days*	*days*	*days*	*days*
0.02	8	3	3	2	2	2	
0.15		6	3	3	2	2	3
0.45		8	6	4	3	3	2
1.00					3	3	2
2.00						8	
CONCENTRATION OF $NaNO_3$							
0.02		5	2	3	2	2	3
0.15		6	2	3	2	2	4
0.45		6	2	4	3	2	
1.00			8	6	3	4	
2.00			8	8	4	4	

The addition of soil and plant extracts proved either indifferent or injurious to growth.

Peptone was undoubtedly a better source of nitrogen for *Bacterium fimi* and another organism apparently belonging to the same group, but it had a very unfavorable influence on the growth of other organisms. Two soils: "Berkeley, California" and "5-B, New Jersey," to which filter paper had been added, were tested for the number of cellulose decomposing bacteria by the dilution method. The medium was the same, except that

$NaNO_3$ was used as the source of nitrogen in one case and peptone in the other (1 gram in each case). *Sp. cytophaga* was the organism to develop at the highest dilution, growth occurring at the following dilutions in the different cases.

	BERKELEY, CALIFORNIA	5-B N.J.
With $NaNO_3$	500,000	50,000,000
With peptone	0	50,000

This effect does not seem to be limited to *Sp. cytophaga*. In a similar experiment with a different soil, an unidentified organism developed at the highest dilution, the results being as follows:

	SOIL: FRESNO, CALIFORNIA
With $NaNO_3$	50,000,000
With peptone	10,000,000

To explain this action of peptone on *Sp. cytophaga*, we may recall that Hutchinson and Clayton (1919) have found that reducing sugars retard or prevent the growth of this organism; the same concentrations of non-reducing sugars do not have any effect. These results have been confirmed in the present work; and they have been found to hold true in the case of another organism (which, similarly to *Sp. cytophaga*, does not attack any carbohydrate, except cellulose). It is possible that the peptone or the products of its decomposition by other organisms also act as reducing agents.

As a result of these experiments, the following medium was selected:

	gm.	
$NaNO_3$	0.50	pH 7.3
K_2HPO_4	1.00	
$MgSO_4 \cdot 7H_2O$	0.50	
KCl	0.50	
$FeSO_4 \cdot 7H_2O$	0.01	
Distilled water	1000	

Five cubic centimeters of this medium were added to each test tube with a piece of filter paper partly immersed.

This medium proved to inhibit the growth of cellulose decomposing fungi, while, at 28°C., a very abundant growth of cellulose-decomposing bacteria could be obtained in a short time. Most of the organisms which were found to be most active under the conditions of our experiments give an abundant growth within thirty-six to seventy-two hours, resulting in disintegration of the paper just at the surface of the medium. On the contrary, *B. fimi* and similar organisms grow within the medium, the part of the paper which is immersed becoming disintegrated. These organisms require seven or eight days for the growth to become apparent.

In all cases 28 to 30°C. has been found the optimum temperature for incubation.

It is especially interesting to report that, with this medium, growth was obtained even when a single cell or very few cells were used for inoculum. A suspension of *Sp. cytophaga* was diluted so as to contain theoretically one organism per cubic centimeter. When 1 cc. was used as inoculum, growth was obtained in 15 per cent of the tubes. Still better results were obtained with the "yellow vibrio" an organism which grows very well on starch agar. A series of dilutions were plated on starch agar, and inoculated at the same time in the test tubes as previously described.

Dilution 6 gave 8 colonies per plate
Dilution 7 gave 1 colony per plate
Tube 6 gave growth in 2 days
Tube 7 gave growth in 4 days

Similar results were obtained with other organisms. It may be considered, therefore, that the method lends itself very well to the enumeration of aerobic cellulose decomposing bacteria.

It also renders the isolation of these organisms easy. The culture to be purified is transferred to a sterile tube of medium and new transfers made as soon as growth is evident. The medium being very specific, the cellulose decomposers are dominant at the beginning of the growth, so that, by a series of dilutions, it is often possible to obtain a pure culture without any

further operations. In many cases, purification can also be obtained by plating on starch agar.

DESCRIPTION OF NEW SPECIES OF CELLULOSE-DECOMPOSING BACTERIA

A large number of species of cellulose-decomposing bacteria have been observed to develop when the medium described above is inoculated with soils in which cellulose has been decomposing under aerobic conditions. The isolation of only a few of these forms has been attempted.

Strictly aerobic forms. By the dilution method, *Spirochaeta cytophaga* was obtained with only one contamination, a very short rod seemingly attached to the filamentous form as if it were parasitizing it. The culture was purified by heating at 65°C. for five minutes; a few of the Spirochetes survived while the short rod was killed. The pure culture thus obtained was in all respects similar to the one described by Hutchinson and Clayton (1919).

All the other forms have been isolated by the dilution method. They will be designated as T, Co, Y, Gr, Calif. For all of them, as well as for the *Sp. cytophaga*, the growth is limited absolutely to the surface of the liquid medium; at this level, the paper is rapidly disintegrated, and breaks into two parts, showing very ragged outlines.

Ammonium salts and nitrates seem to be satisfactory sources of nitrogen; peptone retards the growth considerably. Growth does not occur when the reaction is more acid than pH 6.0. No spores have been observed in any case. Some characters of all these forms are presented in table 4.

The similarity between *Sp. cytophaga* and T is very striking, although they are most certainly different species as indicated by the difference in morphology and rate of cellulose decomposition. Another similarity is found in the following fact. Hutchinson and Clayton reported that some organic substances very much retard the growth of *Sp. cytophaga*. This property seems to be connected with the reducing power of these substances (for instance, maltose is 70 times as effective as sucrose in this respect). In the case of the organism described as T, a few comparative

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TABLE 4

Some differentiating characters of a few strictly aerobic cellulose-decomposing bacteria

ORGANISM AND INVESTIGATOR	OBSERVED IN	MORPHOLOGY	GRAM STAIN	FLAGELLA	GROWTH ON FILTER PAPER IN MINERAL SOLUTION (0.50 GRAM $NaNO_3$ PER LITER)
Spirochaeta cytophaga Hutchinson-Clayton, 1919	England, Germany, France, America (New Jersey and California)	Association of filamentous form 3 x 0.3μ and a sporoid 1.5μ x 1.5 μ	—	0	Yellow pigment outside the fluid after 30 hours incubation
Culture T, 1927	America (New Jersey)	Thin, curved rod 3 to 5 x 0.15μ	—	0	Yellow pigment outside the fluid after 44 hours incubation
Microspira agar-liquefaciens Gray and Chalmers, 1924	England	Curved rod with occasional cocci 2 x 0.6μ	?	1	After 2 to 3 days incubation, the paper breaks in two at the liquid air level
Culture Co, 1926	America (New Jersey and California)	Curved rod 2 to 4 x 0.35μ. Involution forms	—	1	After 36 hours incubation the paper breaks in two at the liquid air level. No pigment
Culture Y, 1926	America (New Jersey)	Curved rod 1–2.5 x 0.3μ. Involution forms	—	1	After 36 hours incubation the paper breaks in two at the liquid alr level. Dark yellow pigment
Culture Gr., 1927	America (obtained from manure)	Curved rod 2 to 3 x 0.35μ. Involution forms	—	1	After 48 hours incubation the paper breaks in two at the liquid air level. Greenish pigment
Culture Calif., 1927	California	Straight rod 2 to 8 x 0.3μ	—	?	After 44 hours incubation the paper breaks in two at the liquid air level. No pigment

ORGANISM AND INVESTIGATOR	GROWTH IN NUTRIENT BROTH	AGAR LIQUEFACTION	GROWTH ON GLUCOSE AGAR	GROWTH ON SUCROSE AGAR	GROWTH ON MALTOSE AGAR
Spirochaeta cytophaga Hutchinson-Clayton, 1919	−	−	−	−	−
Culture T, 1927	−	−	−	−	−
Microspira agar-liquefaciens. Gray and Chalmers, 1924	+	+	Slight	Slight	Slight
Culture Co, 1926	−	−	Abundant, no pigment	Abundant slightly yellow growth	Abundant yellowish
Culture Y, 1926	−	−	Very scanty	Very slight growth	Abundant yellow
Culture Gr., 1927	−	−	−	Slight greenish, turns brown later	Fair and greenish
Culture Calif., 1927	−	−	Very scanty	−	Scanty

ORGANISM AND INVESTIGATOR	GROWTH ON LACTOSE AGAR	GROWTH ON GLYCERIOL AND MANNITOL AGAR	GROWTH ON STARCH AGAR
Spirochaeta cytophaga Hutchinson-Clayton, 1919	−	−	−
Culture T, 1927	−	−	−
Microspira agar-liquefaciens. Gray and Chalmers, 1924	Slight	−	+
Culture Co, 1926	Very heavy growth	−	Very abundant, yellowish
Culture Y, 1926	Fairly abundant yellow		Very abundant, bright yellow, turns brown later
Culture Gr., 1927	Slight	−	Remains thin, greenish
Culture Calif., 1927	Good	−	Good growth

tests with glucose, maltose and sucrose have shown the same effect.

Facultative anaerobe W. A fertile soil (5-B from the soil fertility plots of the New Jersey Experiment Station) was inoculated into test tubes containing a fragment of filter paper and 15 cc. of the following solution: K_2HPO_4, 1.0 gram; $(NH_4)_2SO_4$, 1.0 gram; $MgSO_4 \cdot 7\ H_2O$, 0.5 gram; NaCl, 0.1 gram; $FeSO_4 \cdot 7\ H_2O$, 0.01 gram; distilled water, 1000 grams.

After five or six days, the medium became black due to the reduction of the sulfates. Transfers with increasing dilutions were repeatedly made as soon as the paper began to disintegrate.

TABLE 5

Influence of temperature on the time (days) required for cellulose decomposition to become evident

ORGANISMS	37°C.	28°C.	20°C.
		hours	*hours*
Sp. cytophaga	No growth	72	144
Culture T	No growth	44	84
Culture Co	No growth	36	84
Culture Y	No growth	36	84
Culture Gr	No growth	48	108
Culture Calif	6 days	36	84
Facultative anaerobe W	No growth	120	192

A pure culture was thus obtained without further operations. The culture was also purified by plating on nutrient agar. The colonies obtained from these plates showed evidence of decomposition of the cellulose in nine days when transferred to the medium described above. A few of the characters of the organisms are as follows:

Morphology. Straight rods with occasional coccoid forms. The dimensions vary from 1 to 2μ in length and 0.3 to 0.4μ in width.

In young cultures, in the cellulose medium where the cellulose is submerged, the rods exhibit a typical palisade formation.

No spores have been observed.

Oxygen relations. Growth seems to be the same under aerobic

and anaerobic conditions, but, in all cases, the decomposition of the paper is limited to the part immersed in the medium.

Growth on ordinary media. Small yellow colonies were obtained on nutrient agar within twenty-four hours. Rapid growth was obtained in nutrient broth, gelatin, starch agar, glucose agar and sucrose agar, on the slope as well as in the slab.

In mineral solution containing cellulose, the medium becomes turbid after seven days; the paper retains its shape, but breaks down at the slightest agitation. No gas evolution has been observed in any case with pure culture.

Ammonium salts and nitrates are satisfactory sources of nitrogen, but still more active decomposition of the cellulose is obtained with peptone.

This organism, which grows only between pH 6.4 and pH 8.5, appears to be closely related to the group studied by Kellerman and McBeth.

The influence of temperature on the growth of the organisms studied is indicated in table 5.

The importance of these different groups of organisms in soil products has been discussed elsewhere (Dubos, 1928).

SUMMARY

1. It is shown that a rapid and abundant growth of aerobic cellulose decomposing bacteria is obtained by the use of the following medium which is very simple and very specific: $NaNO_3$, 0.50 gram; K_2HPO_4, 1.0 gram; $MgSO_4 \cdot 7\ H_2O$, 0.50 gram; KCl, 0.50 gram; $FeSO_4 \cdot 7\ H_2O$, 0.01 gram; distilled water, 1000 grams. Five cubic centimeter portions of this medium are introduced into test tubes containing a strip of filter paper partly immersed.

2. The slightly alkaline reaction of the medium (pH 7.5) favors the growth of bacteria while it retards the growth of fungi.

3. The low concentration of nitrogen salts shortens the incubation period of bacteria, since, in almost all cases, cellulose decomposition can be recorded after thirty-six to seventy-two hours at 28°C.

4. Growth is obtained even when only one or very few cells are used for inoculation. This renders possible the use of the

dilution method for determining the numbers of cellulose bacteria and for isolating them in pure culture.

5. Pure cultures of organisms belonging to three different physiological groups have been isolated by means of this method. *a.* Strict aerobes, unable to utilize any of the carbon compounds tested, except cellulose. *b.* Strict aerobes, cellulose decomposers growing well on starch agar, but not on nutrient agar. *c.* Facultative anaerobes, cellulose decomposers, growing well in all ordinary media.

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THE FERMENTATION OF MALTOSE BY BACT. PULLORUM[1]

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Due largely to the work of Smith and Ten Broeck (1915) and Rettger and Koser (1917) maltose fermentation has been quite generally accepted as a criterion for the differentiation of *Bact. pullorum* (Rettger) and *Bact. sanguinarium* (Moore). These workers found that *Bact. sanguinarium* attacked maltose readily, producing acid from this sugar after twenty-four hours incubation. *Bact. pullorum*, on the contrary, did not ferment maltose.

If the literature is examined, however, several references to the fermentation of maltose by *Bact. pullorum* may be found. Krumwiede and Kohn (1917) state in part:

> Maltose fermentation, as noted by Smith and Ten Broeck, differentiates between *B. sanguinarium* and *B. pullorum*. Our results are the same. With prolonged incubation, however, the latter shows irregular evidence of fermentation in from sixteen to forty days. As this sugar is notably subject to hydrolysis, the prolonged incubation rather than a latent ability to ferment may explain these findings.

Spray and Doyle (1921) noted that when serum water was used as the basic medium that maltose was fermented with the production of acid and gas within twenty-four hours. When infusion broth was used as the basic medium no fermentation was observed in five days. May and Goodner (1927) report:

> Although there is, as a rule, a sharp distinction between *S. pullorum* and *S. gallinarum* in the fermentation of maltose, dextrin and dulcitol,

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there may appear slight positive reactions with *S. pullorum* in maltose or dextrin. This is due to the fact that maltose is easily broken down in sterilization and some lots of dextrin contain sufficient impurities to permit some fermentation.

Hendrickson (1927) has recently studied the fermentative properties of *Bact. pullorum* and *Bact. sanguinarium*. He found that when either meat extract or meat infusion broth was used as a basic medium maltose was fermented slowly by *Bact. pullorum*. When serum water was used as a basic medium, however, maltose was rapidly fermented by *Bact. pullorum*. Hendrickson concludes that:

> This work shows that there are but little cultural differences between *Bact. pullorum* and *Bact. sanguinarium*. In fact, aside from the somewhat greater growth vigor on the part of the latter, no characteristic cultural differences were found.

EXPERIMENTAL WORK

In all of the studies quoted above the experiments were conducted using maltose which had been sterilized by heat. It was thought desirable to study the action of *Bact. pullorum* on unheated maltose, since this sugar is readily hydrolyzed by heat. The action of 88 strains of *Bact. pullorum* on maltose has been tested, using meat extract broth and meat infusion broth as basic media. The action of the same strains on maltose has also been tested using serum water as a basic medium.

The cultures of *Bact. pullorum* used in this work were isolated from hens reacting to the agglutination test for bacillary white diarrhea and from baby chicks which had died from bacillary white diarrhea. Each strain isolated from young chicks was found in a separate outbreak of the disease, no two strains being isolated from the same flock. All the cultures when isolated were typical strains of *Bact. pullorum*, producing small, transparent colonies on agar slants, fermenting glucose within twenty-four hours, and not producing acid from maltose, dulcitol, or lactose within seven days. All the cultures were agglutinable in *Bact. pullorum* immune serum.

METHODS

Sterilization of sugars. All the sugar solutions used in this study were sterilized without heat. Ten per cent solutions of the sugars were made up in distilled water and filtered though clean, freshly-burned Berkefeld filters. The filtered solutions were tested for sterility and added aseptically to the previously sterilized broth in sufficient amounts to make a final concentration of 1 per cent. Sugars of the highest purity obtainable were used.

Meat extract broth. Broth was prepared containing 0.3 per cent meat extract and 1.0 per cent peptone. The reaction was adjusted to pH 7.2, the broth heated and filtered. Andrade's indicator was added in the amount of 1 per cent and the medium was tubed. A few drops of mineral oil were placed in each tube to prevent evaporation and the tubes plugged and sterilized.

Meat infusion broth. Fresh meat infusion was sterilized, inoculated with *Bact. coli* and incubated eighteen hours to free the infusion of sugar. The infusion was sterilized and filtered until clear. One per cent peptone was added and the reaction adjusted to pH 7.2. The medium was heated, filtered and 1 per cent Andrade's indicator added. The broth was then tubed and sterilized, each tube being capped with mineral oil.

Serum water. One part of horse serum was added to three parts of distilled water. The medium was divided into two equal parts. To the first part 1 per cent Andrade's indicator was added and the medium was tubed and sterilized. To the second part sufficient HCl was added to bring the reaction to pH 7.2. Andrade's indicator was added and the medium tubed and sterilized.

When testing the action of *Bact. pullorum* on maltose each strain was also inoculated into glucose, dulcitol and lactose broths and the four sugars incubated together. Incubation was continued for sixty-five days at 37°, when the meat extract and meat infusion media were being tested. Readings were made daily for seven days, and thereafter the tubes were examined at intervals of four days. In testing the organisms for their action on maltose in serum water the tubes were incubated for fifteen

days at 37°. Four cultures of *Bact. sanguinarium* were included in the studies as controls.

RESULTS

The reactions of cultures in meat extract broth and meat infusion broth were very similar. In meat extract broth 62 strains produced acid while 26 strains failed to produce acid. The shortest time in which acid production was observed in maltose broth was twelve days. The latest acid production appeared on the forty-fifth day of incubation. In the meat infusion medium 68 strains produced acid while 20 strains showed no indication of having attacked the sugar. The earliest acid production observed was on the tenth day of incubation, while the latest production of acid occurred between the sixtieth and sixty-fifth days. In the majority of strains which produced acid in maltose broth, the initial production of acid became apparent between the twentieth and thirtieth days of incubation.

In both glucose meat extract broth and glucose meat infusion broth all strains produced acid within twenty-four hours. None of the strains were observed to produce acid from dulcitol or lactose.

The 88 *Bact. pullorum* cultures were tested for their ability to ferment maltose when serum water was used as the basic medium. Two lots of serum water were used. The reaction of one lot was left unadjusted, while the reaction of the second lot was adjusted to pH 7.2. Contrary to the results of Spray and Doyle (1921) and Hendrickson (1927) no acid production was apparent after twenty-four hours incubation. The strains were incubated in maltose serum water for fifteen days, and no acid production could be observed during this period, although the tubes were examined daily. This experiment was repeated several times using both horse and beef serum.

EFFECT OF ALKALI ON THE FERMENTATION OF MALTOSE

Bact. pullorum when growing in nutrient broth which does not contain a fermentable sugar slowly alkalinizes the medium. It seemed probable that this production of alkali might explain the

irregular action of *Bact. pullorum* in maltose broth. Sugars, such as maltose, which contain a free aldehyde group are known to be very reactive in alkaline solutions, profound changes occurring even though the amount of alkali present is very small.

In order to determine the amount of alkali produced by *Bact. pullorum* when grown in the absence of a fermentable sugar, the lactose broth cultures of a number of strains of the organism were tested for their reaction. The hydrogen ion concentrations of these cultures were determined electrometrically. The lactose broth cultures had been incubated for forty days at 37° side by side with the maltose broth cultures. The hydrogen ion concentration of broth cultures was originally pH 7.2. The reaction of a composite sample of lactose broth cultures of 10 strains which had not produced acid in maltose broth was pH 7.66. The reaction of a composite sample of lactose broth cultures of 10 strains which had produced acid in maltose broth was pH 8.15. The strains which yielded acid in maltose broth had produced more alkali in lactose broth than had the strains which did not acidify maltose broth. This evidence lends strength to the view that acid production in maltose broth may be due to changes in the sugar induced by alkali production.

An approximately 0.1 N solution of $NaHCO_3$ in distilled water was prepared. This solution was divided into two parts. To one part of the solution sufficient NaOH was added to bring the reaction to pH 8.06. To the second part of the solution sufficient NaOH was added to bring the reaction to pH 8.78. These two alkaline solutions were used to prepare 10 per cent solutions of maltose. A 10 per cent solution of maltose in distilled water was also prepared. The three solutions were sterilized by filtration through clean, freshly burned Berkefeld filters. The solutions were introduced into clean, sterile hard glass tubes and placed in the 37° incubator.

Two lots of meat extract broth having reactions of pH 7.2 and pH 8.4, respectively, were prepared. After the three solutions of maltose had been held for 10 days at 37° they were added to the two lots of broth in sufficient amounts to make a final concentration of maltose of 1 per cent, as shown at top of page 240.

Medium A.	Broth pH 7.2+	Maltose in distilled water.
Medium B.	Broth pH 7.2+	Maltose in solution having a reaction of pH 8.06.
Medium C.	Broth pH 7.2+	Maltose in solution having a reaction of pH 8.78.
Medium D.	Broth pH 8.4+	Maltose in distilled water.
Medium E.	Broth pH 8.4+	Maltose in solution having a reaction of pH 8.06.
Medium F.	Broth pH 8.4+	Maltose in solution having a reaction of pH 8.78.

Twenty strains of *Bact. pullorum* were used to inoculate each of these six media. Ten of these strains had caused a late production of acid in maltose broth in the previous tests, while the other 10 had not caused this production of acid. These 20 strains of pullorum, after being inoculated into the six media, were incubated for fourteen days and observations made daily. No production of acid occurred in fourteen days.

The solutions of maltose after being held at 37° for twenty days were again tested in the same manner as above. The results of this test were quite different from those obtained after the sugar had been held at 37° for 10 days. On the second test there was distinct acid production in media B, C, E and F within twenty-four hours after inoculation. No acid production was noted in media A and D after fourteen days incubation. The exposure of the maltose to the alkaline solutions had so changed the sugar that *Bact. pullorum* was able to produce acid within twenty-four hours.

DISCUSSION

It has been demonstrated that some strains of *Bact. pullorum* will produce acid in maltose broth upon prolonged incubation, even though the maltose is sterilized without heating. However the strains of *Bact. pullorum* used in this study do not attack unheated maltose with the prompt production of acid and gas when serum water is used as a basic medium. The ready fermentation of maltose in serum water which has been observed by investigators using heated maltose may be explained. When

one part of serum is diluted with 3 parts of water and autoclaved the reaction of the resulting medium is approximately pH 8.4. If a solution containing maltose is heated at this reaction there is little doubt that considerable hydrolysis would take place. If the maltose is heated separately and added to the serum water, hydrolysis would take place if the medium were allowed to stand for a period of time before it was inoculated. We have observed changes taking place in maltose at a reaction less alkaline than that of the serum water.

An effort was made to determine to what extent the maltose had become changed when it was kept for twenty days at 37° in a solution having a reaction of pH 8.06. A solution of maltose which had been dissolved in distilled water and held for twenty days at 37° was used as a comparison. A distinct caramelization occurred in the alkaline solution, indicating that polymerization or resin formation had taken place. No difference could be demonstrated in the action of the two solutions on Barfoed's reagent. Using Benedict's quantitatve reagent, no difference could be detected in the reducing power of the two solutions.

There was however a marked difference in the osazones prepared from the two solutions. When the alkaline solution was heated with phenylhydrazine hydrochloride and sodium acetate, glucosazone was formed. No crystals of maltosazone were detected in this preparation. When the maltose dissolved in distilled water was used in the osazone test, many crystals of maltosazone were formed. Mixed with the maltosazone there were a few very small crystals resembling glucosazone.

The optical rotation of the two solutions was determined. The specific rotation of the maltose in distilled water was 124.7, while the specific rotation of the sugar in the alkaline solution was only 98.7. Jolles (1897) states that maltose exposed to N/100 alkali for twenty-four hours at 37° completely loses its rotatory activity.

Broth cultures of *Bact. pullorum* which do not contain a fermentable sugar may reach an alkalinity of pH 8.15. The changes in the maltose, which we have noted above, occurred at a reaction less alkaline than that produced by *Bact. pullorum*. These

changes were sufficient to render the sugar readily fermentable by this organism. We must assume, therefore, that the late acid production in maltose broth by *Bact. pullorum* is not due to the action of the organisms on maltose, but that a slow alkaline hydrolysis of the sugar is effected and that the bacteria act upon the products of this hydrolysis to produce acid.

None of the 88 strains employed in this study have been observed to produce acid from dulcitol. This substance is more reliable in the differentiation of *Bact. pullorum* and *Bact. sanguinarium* than is maltose.

CONCLUSIONS

Bact. pullorum may produce acid in maltose broth upon prolonged incubation, even though the maltose used in the tests has not been heated.

When unheated maltose solution is added to serum water which has been previously sterilized, *Bact. pullorum* does not ferment the sugar within twenty-four hours.

From the data presented above it seems that *Bact. pullorum* does not attack dextro-maltose, but that it may produce acid from some of the products resulting from the alkaline hydrolysis of maltose.

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THE RELATION OF ENCAPSULATED BACILLI FOUND IN METRITIS IN MARES TO ENCAPSULATED BACILLI FROM HUMAN SOURCES[1]

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During the past seven years an extensive study of the causes of sterility in mares has been carried out at the Kentucky Experiment Station. In this work clinical and bacteriological examinations of the reproductive organs of more than 1600 barren mares have been made. Approximately 30 per cent of these mares have been found to be suffering with metritis due to infection. Dimock and Snyder (1923) reported the isolation of streptococci from the cervix and uterus of 26 per cent of a series of barren mares examined. Later Dimock and Snyder (1924) reported the isolation of an encapsulated bacillus from a number of cases of metritis. To this organism they gave the name *Encapsulatus genitalium* (n. sp.). It has been found in approximately 5 per cent of the total number of barren mares examined. Dimock and Edwards (1927) studied a number of strains of encapsulated bacilli recovered from cases of metritis in mares. These cultures were found to be very uniform in cultural characters and serological properties. It was found possible to reproduce typical cases of metritis in normal mares by the introduction into the uterus of pure cultures of encapsulated bacilli recovered from the genital tract of mares.

The present investigation is an attempt to determine the systematic relationship of the encapsulated bacilli recovered from mares to encapsulated bacilli from other sources, especially

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Encapsulatus pneumoniae of Friedländer. Twenty-five strains of encapsulated rods have been isolated from the uteri of infected mares in the thoroughbred studs of Kentucky and Virginia. These cultures were not selected according to their cultural characters, but are representative of the organisms of this group which have been found in mares. In addition 1 strain is included which was isolated from the semen of a stallion at time of service. A number of strains of encapsulated bacilli from diverse sources were obtained from various laboratories and with

TABLE 1

Origin of strains

E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, E17, E18, E19, E20, E21, E22, E23, E24, E25	Isolated from the uteri of infected mares
5176	Semen of stallion after service
F3, F8, F11, F20, F22, Sc	Pneumonia—heart blood, human
F5, F6, F9, F13, F18, F24, F25	Pneumonia—sputum, human
F23	Pneumonia—blood in life, human
F10, F28	Pneumonia—lung tissue, human
F1, F2	Extirpated appendix, human
F21	Extirpated tonsil, human
F26	Chronic colitis—feces, human
F27	Chronic cystitis—urine, human
F15	Mitral regurgitation—sputum, human
F19	Pleuritis—blood in life, human
FR	Stock culture of *B. pneumoniae*, human

these cultures comparative studies have been carried out. The list of strains and the sources from which they were isolated are given in table 1.

CULTURAL CHARACTERISTICS

The cultural characteristics of the equine strains are typical of the *B. mucosus* group. On agar slants there occurs an excessive dirty white to slightly yellow growth which is moist, spreading, glistening and very viscid. Agar colonies are large, raised, round and entire. The bacteria are Gram-negative, non-sporing, non-motile rods which produce wide capsules. The character-

istics of the encapsulated bacilli from human sources were in large part the same as those of the equine strains. Some of these cultures had, however, been cultivated on artificial media for a

TABLE 2

Biochemical reactions

	AMMONIA FORMATION	NITRATE REDUCTION	INDOL PRODUCTION	METHYL RED	VOGES-PROSKAUER	GROWTH IN URIC ACID	GROWTH IN CITRATE	ACTION IN LITMUS MILK*
E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, E17, E18, E19, E20, E21, E22, E23, E24, E25 and 5176	+	+	−	−	+	+	+	AC
F1	+	+	−	+	−	−	+	A
F2	+	+	−	+	−	+	+	A
F3	+	+	−	−	+	+	+	A
F5	+	+	−	−	+	+	+	A
F6	+	+	−	−	+	+	+	AC
F8	+	+	−	+	−	+	+	A
F9	+	+	−	−	+	+	+	A
F10	+	+	−	+	−	+	+	A
F11	+	+	−	−	+	+	+	AC
F13	+	+	−	+	−	−	−	A
F15	+	+	−	−	+	+	+	A
F18	+	+	−	−	+	+	+	A
F19	+	+	−	−	+	+	+	A
F20	+	+	−	−	+	−	−	A
F21	+	+	−	+	−	+	+	AC
F22	+	+	−	−	−	−	−	AL
F23	+	+	−	−	+	+	+	A
F24	+	+	−	+	−	+	+	A
F25	+	+	−	−	+	+	+	A
F26	+	+	−	−	+	+	+	AC
F27	+	+	−	−	+	+	+	A
F28	+	+	−	−	+	+	+	A
FR	+	+	−	+	−	−	−	A
Sc	+	+	−	+	−	+	+	A

* A, acid; AC, acid and coagulation; AL, alkaline.

number of years and had partially or wholly lost their ability to produce wide capsules and as a result, no longer exhibited a mucoid, tenacious growth on agar.

The cultures studied have been subjected to the biochemical tests usually applied to the organisms of the colon-aerogenes group. The strains have been tested for indol production, growth in citrate and uric acid media, nitrate reduction, production of ammonia, action in litmus milk, production of acetyl-methyl carbinol, and response to the methyl red test. A summary of the reactions of the cultures to the various tests is given in table 2.

From table 2 it can be seen that the equine strains form a homogeneous group in relation to the tests employed. All of the 26 strains studied produced ammonia, reduced nitrates, did not produce indol, were methyl red negative and Voges-Proskauer positive, were able to utilize citrate and uric acid, and produced acid and coagulation in litmus milk. The 24 strains of encapsulated bacilli recovered from human sources are rather irregular in their reactions to the tests employed here. All of these cultures produced ammonia and reduced nitrates and none of them produced indol. Several other cultures which were received from various laboratories labelled as Friedländer bacilli but proved to be indol producers were not included in this study.

The reactions of the strains from human sources to the methyl red, Voges-Proskauer, uric acid and citrate tests were varied. It may be noted, however, that the relation between the methyl red and Voges-Proskauer tests is rather close, that is, where one of these tests is positive the other is negative. This is true in the case of every strain except one, F22. This strain is apparently non-saccharolytic. This relation between the methyl red and Voges-Proskauer tests is analogous to the condition found in the colon-aerogenes group.

The relation of the methyl red and Voges-Proskauer tests to the uric acid and citrate tests, which usually exists in the colon-aerogenes group, has not been demonstrated with these strains of encapsulated bacilli from human sources. The equine strains do display this relation, however, giving the reactions considered typical of the soil types of *Bact. aerogenes*. In litmus milk the equine strains produced acid and coagulation in twenty-four hours. After several days the litmus was reduced, there was a marked extrusion of whey and the curd was shot with gas. With

one exception the Friedländer bacilli produced acid in litmus milk. The strain which did not produce acid is a non-lactose fermenting organism. This culture produced sufficient alkalinity in milk to bring the casein into solution. There was no evidence of digestion in any of the milk cultures after they had been incubated at 37°C. for twenty-one days.

FERMENTATIVE REACTIONS

The fifty strains of encapsulated bacilli were tested for their ability to form acid and gas from a number of fermentable substances. The basic medium used to test for fermentation was nutrient broth containing 1 per cent peptone and 0.3 per cent meat extract adjusted to reaction of pH 7.2. Andrade's indicator was added in the amount of 1 per cent. Durham fermentation tubes were used to observe gas formation. The basic medium containing Andrade's indicator was tubed and sterilized in the autoclave. The fermentable substances were sterilized by passage through a Berkefeld filter and added to the previously sterilized broth in sufficient amount to make a final concentration of 1 per cent. The action of the organisms on the various fermentable substances is given in table 3.

An examination of table 3 shows that the 26 strains of equine origin were identical in their action upon the 18 fermentable substances employed in this study. They fermented glucose, levulose, galactose, lactose, sucrose, glycerol, mannitol, sorbitol, arabinose, xylose, raffinose, maltose, adonitol, salicin, dextrin and soluble starch with the production of acid and gas. Dulcitol and inulin were not attacked. Thirteen of the Friedländer strains possess the same fermentative characters as this group of equine strains. One Friedländer culture, F18, attacked the same substances as the equine strains, but did not produce a perceptible amount of gas from any of them. Another Friedländer strain, F20, resembled the equine strains in its fermentative properties except that it produced acid without gas from sucrose.

Strains F10 and F27, in addition to producing acid and gas from the substances attacked by the equine strains, fermented dulcitol with acid and gas formation. Strain Sc attacked the

TABLE 3

	GLUCOSE	LEVULOSE	GALACTOSE	LACTOSE	SUCROSE	GLYCEROL	DULCITOL	MANNITOL	SORBITOL	ARABINOSE	XYLOSE	HARTINOSE	MALTOSE	ADONITOL	SALICIN	INULIN	DEXTRIN	SOLUBLE STARCH
E1, E2, E3, E4, E5, E6, E7, E8, E9, E10, E11, E12, E13, E14, E15, E16, E17, E18, E19, E20, E21, E22, E23, E24, E25, 5176	AG	AG	AG	AG	AG	AG	0	AG	AG	AG	AG	AG	AG	AG	AG	0	AG	AG
F3, F5, F6, F8, F13, F15, F19, F21, F24, F25, F26, F28	AG	AG	AG	AG	AG	AG	0	AG	AG	AG	AG	AG	AG	AG	AG	0	AG	AG
F2	AG	AG	AG	AG	0	AG	0	AG	AG	AG	AG	AG	AG	AG	AG	0	AG	AG
F20	AG	AG	AG	A	A	AG	0	AG	AG	AG	AG	AG	AG	AG	AG	0	AG	AG
F23	AG	AG	AG	0	AG	AG	0	AG	AG	AG	AG	AG	AG	AG	AG	0	AG	AG
F10, F27	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	0	AG	AG
Sc	A	A	A	A	A	A	A	AG	AG	A	A	A	A	A	A	0	AG	AG
F1	A	A	A	A	0	A	0	A	A	A	A	A	A	A	A	0	A	A
F11	A	A	A	0	A	0	0	A	0	A	A	0	0	0	0	0	0	0
F18	A	A	A	A	A	A	0	A	A	A	A	A	A	A	A	0	A	A
F22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

A, acid; AG, acid and gas; 0, no acid or gas.

same substances as strains F10 and F27, but was irregular in its gas production. Gas formation by this strain usually occurred only after six to eight days incubation.

Strain F2 produced acid and gas from all the fermentable substances with the exception of sucrose, dulcitol, and inulin. It is set off from the equine strains by its failure to ferment sucrose. Strain F1 attacked the same substances as strain F2 but did not produce gas from any of them. Strain F23 fermented sucrose with acid and gas production but did not attack lactose. Strain F11 produced acid from glucose, levulose, galactose, sucrose, mannitol, arabinose and xylose. The other substances were not attacked. Strain F22 is apparently non-saccharolytic.

Clairmont (1902) in studying the encapsulated bacilli, found that when fermentation occurred both acid and gas were produced in glucose, lactose and sucrose alike, with the exception of 2 strains which fermented sucrose with acid formation but failed to form gas. Perkins (1904) divided the mucosus group into three types according to their action on glucose, lactose and sucrose. Type I was composed of organisms fermenting all these sugars with acid and gas formation; group 2 of organisms fermenting glucose and sucrose with acid and gas, and group 3 of organisms fermenting glucose and lactose with acid and gas. Coulter (1917) found that all the strains of Friedländer bacilli which he studied formed acid and gas from glucose and sucrose, but failed to attack lactose. Fitzgerald (1914) reported that fermentation reactions within the mucosus group were highly variable and could not be relied upon as a basis of classification. Small and Julianelle (1923) studied a number of strains of the mucosus group and found that certain strains did not attack any of the sugars used in their work. They state that fermentation reactions are not a satisfactory basis for classification.

The strains of encapsulated bacilli employed in this study have been shown to be divisible into several groups on the basis of their action upon the fermentable substances tested. However, these groupings are not substantiated by the action of the organisms toward the other biological reactions (i.e., methyl red, Voges-Proskauer, citrate, etc.) nor, as will be shown later, do they cor-

respond to the groupings established by the serological tests. One unusual fact is brought out by the biochemical tests. This is the uniformity displayed by the equine cultures. It is unusual to find such a large number of strains of encapsulated bacilli from a single source which are identical in their biochemical characteristics.

SEROLOGICAL REACTIONS

The literature regarding the serological properties of the Friedländer group is as confusing as it is voluminous. The recent work of Julianelle (1926, 1926a, 1926b,) has gone far in explaining the contradictory results obtained by various workers. He has demonstrated that specificity in the Encapsulatus group resides in the capsule. Toenniessen (1914) suggested that the capsular substance was antigenic in character and that Friedländer bacilli were capable of exhibiting two antigenic complexes, one residing in the capsule and the other in the endoplasm of the bacterium. Toenniessen (1921) later demonstrated that the capsule of these organisms was a galactan. Heidelberger, Goebel and Avery (1925) derived a nitrogen-free polysaccharide from Friedländer bacilli which was capable of giving positive precipitin tests with homologous immune serum. This polysaccharide was comparable to the soluble specific substance of pneumococcus and was active in a dilution of 1:4,000,000. Julianelle (1926) by applying these facts to the classification of Friedländer strains has established four serological groups among the encapsulated bacilli. These he has designated as types A, B, and C, and Group X. Types A, B and C are specific while group X is composed of a number of heterologous strains.

In the present investigation immune serums have been prepared from several of the strains studied. Using these serums, agglutination tests, precipitin tests and agglutinin absorption tests have been performed.

METHODS.

Immunization. Rabbits were used to prepare immune serums. They were injected intravenously with heat-killed cultures of the

encapsulated bacilli. Injections were made at intervals of three days. The first dose was 0.25 cc.of an agar slant culture suspended in 10 cc. of normal saline solution. The dose was gradually increased to 1.0 cc. Trial bleedings were made from time to time to determine the titer of the serums. Satisfactory titers were usually attained after 12 to 15 injections. The normal serum used in the experiments consisted of the pooled serums of the rabbits bled before immunization was begun.

Agglutination. In preparing the agglutination antigens agar slant cultures incubated for twenty-four hours at 37°C. were suspended in carbolized saline and the turbidity adjusted to 0.5 on the McFarland nephelometer scale. The agglutination tests were set up in dilutions ranging from 1:10 to 1:2000. The tests were incubated forty-eight hours at 37°C. before being read. In agglutinin absorption tests the serums were diluted 1:5 with suspensions of homologous organisms of varying concentrations in order to determine the absorptive dose. The smallest amount of homologous organisms which completely absorbed the agglutinins for itself was used as the absorbing dose in subsequent tests. Absorptions were carried out at 37°C. for two hours and in the icebox overnight.

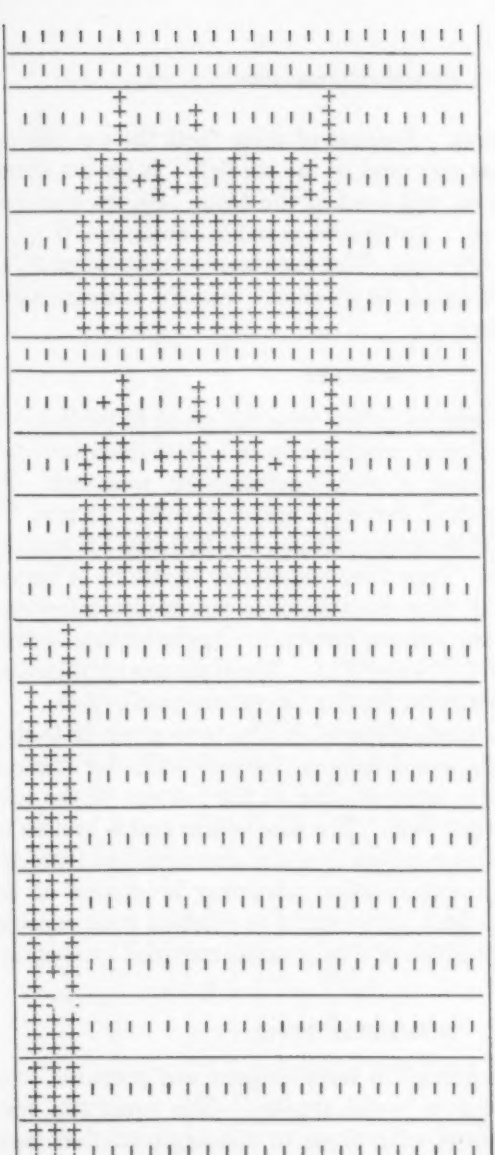
Precipitin test. The antigens for the precipitin tests were prepared in two ways. The first method was that used by Small and Julianelle (1923). Broth cultures of the encapsulated bacilli were incubated at 37°C. for thirty days, made up to the original volume with sterile distilled water and centrifuged. The supernatant fluid was filtered through Berkefeld filters to remove the bacilli completely. The resulting clear fluid was placed in sterile tubes and stored in the ice chest until used.

In the second method twenty-four-hour agar slant cultures were suspended in normal saline and autoclaved at 15 pounds for fifteen minutes. The suspension was centrifuged and the supernatant fluid filtered through Berkefeld filters. The clear filtrate was used as antigen in the precipitin tests. Both methods gave very satisfactory antigens. However, the antigen derived from the agar cultures was much more concentrated and had less tendency to give group reactions than did the antigen derived

TABLE 4

Agglutination reactions

ANTIGENS	DILUTIONS OF ANTISERA																					Normal serum
	E1					F6					Sc					F8						
	1:10	1:20	1:50	1:100	1:200	1:10	1:20	1:50	1:100	1:200	1:10	1:20	1:50	1:100	1:200	1:10	1:20	1:50	1:100	1:200	1:10
E1	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−
E2	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−
E3	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	−	−	−	−	−	−	−	−	−	−	−
E4	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−
E5	++++	++++	++++	++++	++++	++++	++++	++++	++++	+	−	−	−	−	−	−	−	−	−	−	−
E6	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	−	−	−	−	−	−	−	−	−	−	−
E7	++++	++++	++++	++++	−	++++	++++	++++	+++	−	−	−	−	−	−	−	−	−	−	−	−
E8	++++	++++	++++	++++	++	++++	++++	++++	++++	+	−	−	−	−	−	−	−	−	−	−	−
E9	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−
E10	++++	++++	++++	++++	++++	++++	++++	++++	++	−	−	−	−	−	−	−	−	−	−	−	−
E11	++++	++++	++++	++++	+	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−
E12	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−
E13	++++	++++	++++	++++	+	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−	−
E14	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−	−
E15	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−
E26	++++	++++	++++	++++	++	++++	++++	++	−	−	−	−	−	−	−	−	−	−	−	−	−
E17	++++	++++	++++	++++	+	++++	++++	++++	+	−	−	−	−	−	−	−	−	−	−	−	−
E18	++++	++++	++++	++++	+	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−	−
E19	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−
E20	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−
E21	++++	++++	++++	++++	−	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−	−
E22	++++	++++	++++	++++	++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−	−	−
E23	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−
E24	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−
E25	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	−	−	−	−	−	−	−	−	−	−	−
5176	++++	++++	++++	++++	++	++++	++++	++++	++	−	−	−	−	−	−	−	−	−	−	−	−


F6
F26
F16
F3
F5
F8
F9
F15
F18
F19
F20
F21
F23
F24
F25
F28
8c
F1
F2
F10
F11
F13
F22
F27

from old broth cultures. Because of these facts the antigen derived from agar slants was used in the studies. It gave a strong reaction when 0.001 cc. was used in the tests. Serum was used in the amount of 0.1 cc. Antigen was used in amounts of 0.5, 0.1, 0.05, 0.02, 0.01 and 0.001 cc. The tests were incubated at 50°C. for one hour, read, placed in the ice chest overnight and read again the following morning.

RESULTS

The results of the agglutination tests are given in table 4. It is evident that the strains under study can be divided into two main types on the basis of their agglutination reactions. All the equine cultures are of the same agglutinative type and fall into type 1. Three Friedländer strains, F6, F26 and FR are also included in this type. Type 2 is composed of 14 strains of human origin, the majority of them isolated from respiratory infections. Seven of the 50 strains remain untyped. Agglutinating serums were prepared from F1, F10 and F13. These serums agglutinated only the homologous strains, leaving the others unaffected.

The agglutination which occurred in the tests given in table 4 was type specific. The reactions were characteristic, the clumped bacilli forming a large, compact disc at the bottom of the tube with a clear supernatant fluid. The disc is firm and is broken up with difficulty. Such a reaction is easily distinguished from the fine, granular, group agglutination occurring in strains of the Encapsulatus group which have ceased to produce wide capsules or which have been denuded of this capsular material by chemical agents.

PRECIPITIN TESTS

In performing the precipitin tests antigen was prepared from each of the 50 strains of bacilli studied. The precipitin tests gave groupings identical with those of the agglutination tests. The antigens prepared from twenty-four-hour agar slant cultures gave highly specific reactions; only in two instances were there any indications of non-specific reactions and these were very slight. In table 5 are given the precipitin tests performed with

TABLE 5

Precipitin tests

ANTIGENS	E1 SERUM							Sc SERUM						
	Dilutions of antigens							Dilutions of antigen						
	0.5	0.1	0.05	0.02	0.01	0.001	Control	0.5	0.1	0.05	0.02	0.01	0.001	Control
E1	−	+++	++++	++++	++++	++++	−	−	−	−	−	−	−	−
E2	+	++	++++	++++	++++	+++	−	±	−	−	−	−	−	−
E5	−	+	+++	++++	++++	++++	−	−	−	−	−	−	−	−
E10	−	++	++++	++++	++++	++++	−	−	−	−	−	−	−	−
E22	−	+	++	++	+++	++++	−	−	−	−	−	−	−	−
F6	−	±	+	++	+++	++++	−	−	−	−	−	−	−	−
F26	−	+	+++	++++	++++	++++	−	−	−	−	−	−	−	−
F3	−	−	−	−	−	−	−	−	++	++	+++	++++	++++	−
F5	±	−	−	−	−	−	−	±	+++	+++	++++	++++	+	−
F8	−	−	−	−	−	−	−	+	++	++	+++	++++	+	−
F15	−	−	−	−	−	−	−	−	+	++	+++	++++	++++	−
F18	−	−	−	−	−	−	−	−	±	+	++	+++	++++	−
F28	−	−	−	−	−	−	−	±	+	++	+++	++++	++++	−
Sc	−	−	−	−	−	−	−	−	±	++	+++	++++	++++	−
F13	−	−	−	−	−	−	−	−	−	−	−	−	−	−
F22	−	−	−	−	−	−	−	−	−	−	−	−	−	−

++++, indicates heavy, compact disc precipitate; +++, compact disc precipitate; ++, slight precipitate; +, marked turbidity; ±, very slight turbidity; −, no reaction.

16 of the strains, using one serum representative of each of the two main types. These reactions are typical of those obtained with the remainder of the strains. It may be seen in table 5 that the reactions were highly specific. When the antigen was added

TABLE 6

Agglutinin absorption

Serum of rabbit immunized to E1

ABSORBING ANTIGEN	AGGLUTINATING ANTIGEN	1:10	1:20	1:40	1:100	1:200
–*	E1	++++	++++	++++	++++	++++
E1	E1	–	–	–	–	–
–	E2	++++	++++	++++	++++	++++
E2	E2	–	–	–	–	–
E2	E1	–	–	–	–	–
–	F6	++++	++++	++++	++++	++++
F6	F6	–	–	–	–	–
F6	E1	–	–	–	–	–
–	F26	++++	++++	++++	++++	++++
F26	F26	–	–	–	–	–
F26	E1	–	–	–	–	–
–	F3	–	–	–	–	–
F3	F3	–	–	–	–	–
F3	E1	++++	++++	++++	++++	++++
–	Sc	–	–	–	–	–
Sc	Sc	–	–	–	–	–
Sc	E1	++++	++++	++++	++++	++++
–	F11	–	–	–	–	–
F11	F11	–	–	–	–	–
F11	E1	++++	++++	++++	++++	++++

* – indicates unabsorbed serum.

to the serum a heavy precipitate occurred in the tubes containing the higher concentrations of antigen. Precipitation in the tubes containing the smaller amounts of antigen was slower and appeared only after incubation of the tests. The heavy precipitate in the tubes containing the stronger concentrations of

antigen was totally or partially redissolved on incubation, due probably to the presence of an excess of antigen.

In this series of tests there were only two instances of non-specific precipitation. A slight turbidity developed with antigen derived from strain E2 in the presence of serum prepared from

TABLE 7

Agglutinin absorption

Serum of rabbit immunized to F6

ABSORBING ANTIGEN	AGGLUTINATING ANTIGEN	1:10	1:20	1:40	1:100	1:200
–	F6	++++	++++	++++	++++	++++
F6	F6	–	–	–	–	–
–	F26	++++	++++	++++	++++	++++
F26	F26	–	–	–	–	–
F26	F6	–	–	–	–	–
–	FR	++++	++++	++++	++++	++++
FR	FR	–	–	–	–	–
FR	F6	–	–	–	–	–
–	E1	++++	++++	++++	++++	++++
E1	E1	–	–	–	–	–
E1	F6	–	–	–	–	–
–	F3	–	–	–	–	–
F3	F3	–	–	–	–	–
F3	F6	++++	++++	++++	++++	++++
–	Sc	–	–	–	–	–
Sc	Sc	–	–	–	–	–
Sc	F6	++++	++++	++++	++++	++++
–	F11	–	–	–	–	–
F11	F11	–	–	–	–	–
F11	F6	++++	++++	++++	++++	++++

strain Sc. Likewise a faint cloudiness occurred when F5 antigen was incubated with E1 serum. These group reactions occurred only in the highest concentration of the antigens and were probably due to the presence of small amounts of group antigens in preparations derived from these 2 strains.

AGGLUTININ ABSORPTION

In the agglutinin absorption tests every one of the 50 strains was used as antigen to absorb each of the immune serums. In table 6 are given the reactions of several of the strains when used as antigen to absorb E1 serum. From table 6 it may be seen

TABLE 8

Agglutinin absorption

Serum of rabbits immunized to Sc

ABSORBING ANTIGEN	AGGLUTINATING ANTIGEN	1:10	1:20	1:40	1:100	1:200
–	Sc	++++	++++	++++	++++	–
Sc	Sc	–	–	–	–	–
–	F3	++++	++++	++++	–	–
F3	F3	–	–	–	–	–
F3	Sc	–	–	–	–	–
–	F8	++++	++++	++++	++++	–
F8	F8	–	–	–	–	–
F8	Sc	–	–	–	–	–
–	E1	–	–	–	–	–
E1	E1	–	–	–	–	–
E1	Sc	++++	++++	++++	++++	–
–	F6	–	–	–	–	–
F6	F6	–	–	–	–	–
F6	Sc	++++	++++	++++	++++	–
–	F26	–	–	–	–	–
F26	F26	–	–	–	–	–
F26	Sc	++++	++++	++++	++++	–
–	F11	–	–	–	–	–
F11	F11	–	–	–	–	–
F11	Sc	++++	++++	++++	++++	–

that strain E2 completely absorbed the agglutinins for E1 from E1 serum. F6 and F26 also effected a complete removal of agglutinins for the homologous strain. The remainder of the equine strains, as well as strain FR, completely exhausted the serum of agglutinins. None of the strains of group 2 removed

the agglutinins from E1 serum, nor were any of the untyped strains able to absorb the agglutinins for the homologous strain.

In table 7 are given the reactions of several of the strains of various types when used as absorbing antigen with serum derived from F6. It is apparent that the results obtained with F6 serum are identical to the results obtained through the use of E1 serum in the absorption tests. All of the strains of group 1 exhaust the serum of agglutinins for the homologous culture. The strains of group 2 and the untyped strains fail to bring about a removal of agglutinins from F6 serum.

In table 8 are given the reactions of several strains of the different types with serum derived from Sc. In this case the strains of group 1 and the untyped strains are unable to absorb the agglutinins from the serum, while the strains of group 2 completely remove them.

The groupings observed by agglutination and precipitin tests have been further substantiated by the agglutinin absorption reaction. It is a significant fact that when a strain exhibited specific agglutination with a serum it was able to exhaust that serum of agglutinins. This was found to be true in every case studied.

THE PRODUCTION OF METRITIS IN MARES BY ENCAPSULATED BACILLI

Dimock and Edwards (1927), on the basis of earlier experiments, reported that metritis in mares was readily reproduced through the introduction into the uterus of encapsulated organisms from equine sources. However, they were unable to reproduce the infection through the use of encapsulated bacilli recovered from human sources. Since the time that this statement was made further inoculation experiments have been carried out and a larger number of strains tested.

In testing the ability of encapsulated bacilli to produce metritis, pure cultures of the organisms are introduced into the uterus in gelatin capsules. Every possible precaution is taken not to injure the mucosa of the genital tract and not to introduce microörganisms other than those being tested. The mares are examined repeatedly before inoculation to insure the absence of infection

of the genital tract. Examinations are made at regular intervals after the introduction of the bacilli into the uterus. Both clinical appearances and bacteriological results are used to determine the production of metritis.

The technique used in making the examinations is that described by Dimock and Synder (1923). The external genitals are thoroughly washed with soap and water and bathed in antiseptic solution. The vagina is opened with a vaginal speculum and the mucous membranes of the vagina and *os uteri* are examined for congestion and inflammation. The presence or absence of exudate is noted. A platinum loop in a long holder is flamed and passed through the *os uteri*. It is carefully withdrawn and rubbed over the surface of agar slants. Using such a procedure the culture tubes show no evidence of bacterial growth when normal mares are examined. When mares suffering with metritis due to encapsulated organisms are examined the bacilli are recovered in pure culture on the agar slants.

When organisms which are not capable of producing metritis are introduced into the normal uterus they disappear very rapidly. Even though enormous numbers of bacilli are introduced they cannot be recovered on cultures after forty-eight to seventy-two hours. The appearance of the *os uteri* and the mucous membranes of the vagina remain unchanged.

When organisms which are capable of producing metritis are introduced into the uterus the results are quite different. Under these conditions an inflammation of the *os uteri* and the vaginal mucous membranes becomes apparent within forty-eight hours. There is a marked discharge of a muco-purulent exudate from the uterus. As the infection progresses the external folds of the *os uteri* often become enlarged and edematous. The organisms which were introduced, instead of disappearing rapidly, can be recovered in large numbers when cultures are taken from the *os uteri*. Such an infection, after becoming established, usually persists indefinitely.

Following the procedure outlined above we have found it possible not only to produce metritis in mares by the use of organisms recovered from this condition but also by the use of strains

isolated from human sources. Metritis was produced when strains F6 and F26 were introduced into the uterus. These two organisms belong to type 1 and are indistinguishable from the equine cultures. Strain Sc has also been found capable of producing metritis. This culture belongs to type 2 and can be differentiated from the equine cultures by serological methods. It is apparent that the production of metritis in mares cannot be relied upon as a criterion to distinguish the equine strains from cultures of human origin.

Baerthlein (1918) and Toenniessen (1921) have demonstrated that the virulence of Friedländer bacilli for the laboratory animals is dependent upon capsule formation. Cultures producing large amounts of capsular material are extremely virulent for the laboratory animals, while strains which have lost this property are avirulent. We have found that the same conditions apply to the production of metritis in mares. Only those organisms which are vigorous capsule producers are infective when placed in the uterus. Organisms which have totally or partially lost the property of producing wide capsules and which do not exhibit a stringy, mucoid growth on artificial media are incapable of initiating pathological changes in the uterus. Strains which have lost their infective properties may often be restored to full virulence by a process of artificial selection. Such cultures may regain their virulence by continued plating on glucose agar and selection of the colonies exhibiting the most mucoid, viscid growth.

DISCUSSION

In studying 50 strains of encapsulated bacilli it has been found that the biochemical tests employed were of little or no value for the purpose of classification. The one fact worthy of note is the uniformity of the equine cultures in regard to the biochemical tests employed. However, many of the cultures recovered from human sources gave the same reactions. The groupings established by the biochemical reactions of the strains do not agree with the groups established by the serological tests.

The results of the serological tests confirm the recent work of Julianelle (1926). The strains from human sources which we

have used in this study are largely the same as those employed by Dr. Julianelle in his work, and the groupings obtained are almost identical. The organisms which constitute type 1 in this study are identical with the type B of Julianelle, while type 2 is synonomous with Julianelle's type A. The incidence of the two types is reversed in the two studies. Julianelle found only 6 members of the type here designated as type 1. The majority of our strains fell into this type. It is worthy of note that most of the strains of respiratory origin are included in type 2. Of 29 strains in type 1 only one was isolated from a respiratory disease, while the source of one was unknown.

The titres of the serums used in this study were higher than those used by Julianelle. He states that serums of higher titre are to be avoided since they will contain non-specific group agglutinin. The serums used in this study contained group agglutinin to a marked degree. This is not perceptible in the agglutination tests because all the organisms employed as antigens in these tests were producing heavy capsules at the time the tests were done. Later, several of the strains lost the property of producing heavy capsules. They were then acted upon by the group agglutinin. The agglutination of these strains was no longer type specific, but they were acted upon both by type 1 serum and type 2 serum. This recalls the results of Porges (1905), Streit (1906) and Beham (1911). These workers found that bacilli which had been denuded of their capsules by chemical agents or by cultural methods became readily agglutinable.

Griffith (1923), Reimann (1925), and Amoss (1925) have demonstrated that the virulence and type specificity of pneumococci are dependent upon capsule formation. It is evident that the same conditions exist in the Encapsulatus group.

The results obtained in the agglutinin absorption studies were unusually regular. In every instance in which a strain gave type specific agglutination with a serum, it was able to completely exhaust that serum of agglutinins for its homologous strain. No sub-groups were established by the absorption tests. The serums were of comparatively low titre and it is possible that slight

differences in antigenic structure would not be apparent under these conditions.

So far as we have been able to determine the equine strains are of uniform antigenic structure and are identical with certain of the cultures of human origin, namely F6, F26 and FR.

Only two serological types have been established in this study. Julianelle (1926) demonstrated a third type composed of 3 strains. It is probable that if a larger number of strains were studied other types would be found.

CONCLUSIONS

Of the 50 strains of encapsulated bacilli studied 43 are divisible into two serological types. Seven of the strains remain untyped.

The 25 cultures recovered from cases of metritis in mares all belong to a single, well defined type. Three cultures of human origin also fall into this type and are indistinguishable from the equine strains by any of the methods employed. The second type is composed of fourteen strains of human origin.

The type specificity and virulence of encapsulated bacilli are dependent upon capsule formation.

The writer wishes to express his indebtedness to Dr. W. W. Dimock for his interest, coöperation and helpful suggestions throughout the course of this work. Our thanks are also due Dr. J. C. Small, Dr. L. A. Julianelle and Dr. L. F. Rettger for forwarding cultures of encapsulated bacilli.

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SOME CONDITIONS AFFECTING THE PRODUCTION OF GELATINASE BY PROTEUS BACTERIA[1]

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INTRODUCTION

It has been shown by previous investigators that the production of proteolytic enzymes by bacteria is affected by changes in the composition of the medium in which the organisms are grown, by variations in the acidity of the culture, and by the amount of oxygen available to the bacteria during growth.

Brunton and Macfadyen (1889) concluded that a stronger enzyme, capable of liquefying gelatin, was produced by bacterial cultures grown in meat broth media than in nutrient gelatin. Fermi (1892) showed that little or no gelatin-liquefying enzyme was obtained in cultures grown in his protein-free media while considerable was formed in nutrient gelatin.

Abbott and Gildersleeve (1903) stated that production of gelatinase was better in alkaline culture media than in acid culture media. At the 1919 meeting of the Society of American Bacteriologists, Clark (1920) reported an optimum production of gelatinase by Proteus bacteria in media near the pH of optimum activity of the enzyme and a decrease in production in media of original reaction of pH 6.5.

[1] Published by permission of the Surgeon General, U. S. Public Health Service.

Liborius (1886) showed a greater production of gelatin-liquefying enzyme in cultures exposed to oxygen than in cultures from which oxygen was excluded. Clark (1920) reported that anaerobiosis shuts off the production of gelatinase almost completely.

In the present paper, the investigations described include a description of the method of Palitzsch and Walbum (1912) as modified by Clark (1919) for the estimation of gelatinase; the quantitative data concerning the reports given at the meetings of the American Society of Bacteriologists by Clark (1919–1920)[3] and by Clark and Merrill (1925), and in addition, further studies on the effect of aerobic and anaerobic conditions on the production of gelatinase, and the development of a synthetic medium in which a good production of gelatinase by Proteus[4] is obtained and so controlled that uniform results can now be obtained.

EXPERIMENTAL

Cultures

For the study of the production of gelatinase by Proteus, strains of this group were obtained from L. A. Rogers, Bureau of Dairying, United States Department of Agriculture. Most of the work was carried on with the strain designated acx. The description determined by Rogers and co-workers for acx, is as follows: Isolated from spring water, it gives typical growth on agar, digests milk, liquefies gelatin, and with glucose gives off carbon dioxide and no hydrogen (ratio = ∞) gas, and forms acid. It does not ferment lactose. The gas obtained in fermentation with glucose was small and only recovered by the vacuum method. Proteus strains other than acx were used in the preliminary studies (Clark, 1920) as indicated in the charts.

[3] The preliminary studies (Clark, 1920) were made with the support of the Dairy Division, now the Bureau of Dairying, United States Department of Agriculture.

[4] Throughout this paper the term Proteus will be used to signify Proteus bacteria.

Estimation of gelatinase activity

The activity of gelatinase is usually determined by the rate of liquefaction of gelatin by the enzyme. The methods for determination of gelatin liquefaction are reviewed by Marras (1914). The procedure used by Fermi (1892), Palitzsch and Walbum (1912), Dernby (1918) and others was modified by Clark (1920) as follows. Melted gelatin and the medium containing the enzyme were mixed and after a period of digestion, a portion of the mixture was transferred to a test tube, and cooled in ice water. The time required for the mixture to set to a selected consistency was marked by a stop watch. This procedure is to be preferred to that of Palitzsch and Walbum, who plunged their mixture into ice water for ten minutes. To the consistencies observed, they gave arbitrary numbers which have no well defined meaning, while Clark's procedure calls for but one consistency.

Clark found empirically that the following equation could be used,

$$K = \frac{\log t_s - C}{T}$$

Here C is the logarithm of the constant time in minutes required for the gelatin control without enzyme to set to the selected state, t_s is the time in minutes required for the digested mixture to set in ice water, T is the time in minutes of incubation of the gelatin-enzyme mixture and K a constant, characterizing the enzyme strength. For this equation to be applicable, several conditions must be fulfilled as shown by Palitzsch and Walbum (1912). The temperature of incubation of the mixture must be constant. The concentration of gelatin must be constant. The pH of the gelatin mixture for the chilling test must be the same and such that the undigested gelatin sets well in ice water. Patten and Johnson (1919) have shown that the setting of gelatin is influenced by the pH of the medium and that within certain limits this hydrogen ion concentration effect is probably reversible. We chose pH 6.2 for the chilling test. The K values in comparative measurements must be determined with a single

batch of gelatin and if the mixtures of gelatin and enzyme are incubated at various pH values, each must be readjusted to the same value for the chilling test and brought to the same concentration of gelatin calculated as unattacked gelatin.

Accordingly, preliminary tests are made with indicators or electrode to determine the proportions of mixture and acid or alkali which must be used, in order that the mixture may be adjusted rapidly after the incubation period.

The stock gelatin contained 10 per cent Difco gelatin (washed free of calcium) 0.1 M H_3BO_3, and 0.1 M Na_2HPO_4 and was preserved with 0.5 per cent phenol. Before use 100 cc. of the melted stock gelatin was transferred to an Erlenmeyer flask, sufficient N sodium hydroxide solution added to bring to the desired pH, and distilled water then added to make the total volume 150 cc. This gelatin stock "solution" diluted from 100 cc. of the stock gelatin to 150 as just described we will designate as gelatin "solution" A.

An example of the procedure for estimation of gelatinase is as follows:

Proteus was grown in a medium made of 2.0 per cent Difco peptone, 1 per cent $Na_2HPO_4 \cdot 2H_2O$[5] and 2 per cent glucose adjusted to pH 7.0 with N hydrochloric acid solution. Fifty cubic centimeters of medium in a 1000-cc. Erlenmeyer flask, stoppered with a cotton plug to provide sufficient aeration, was inoculated with the bacteria and incubated at 30°. Within forty-eight hours considerable gelatinase was produced. To determine the gelatinase, a preliminary test was made to determine the number of cubic centimeters N sodium hydroxide solution required in the gelatin "solution" A so that on mixing with the culture solution, the final reaction of the mixture would be pH 8.4. In this case 5 cc. N sodium hydroxide solution was used in the gelatin "solution" A.

Twenty-five cubic centimeters of the bacterial culture solution were then well mixed with 100 cc. of gelatin "solution" A at 30° in a 300 cc. Erlenmeyer flask and the mixture incubated at 30°

[5] One per cent $Na_2HPO_4 \cdot 2H_2O$ was added to the culture medium to control the pH of the culture.

in a water bath. (The phenol prevented any further growth of the bacteria.)

Five cubic centimeters of the mixture were removed by means of a pipette at varying intervals and transferred to tests tubes. Five-tenths N hydrochloric acid solution was added to bring the reaction of the test mixture to pH 6.2 and the whole plunged into ice water. The test tube was gently shaken. A stop watch marked the time required to bring the mixture to a selected

TABLE 1

$$K = \frac{\log t_s - C}{T}$$

t_s	$\log t_s$	T	C ESTIMATED	K
minutes		*minutes*		
0.67	−0.17	8	−0.40	0.0288
0.75	−0.13	13		0.0208
1.08	+0.03	21		0.0205
2.00	+0.30	32		0.0219
3.83	+0.58	38		0.0258
Average				0.0236
0.67	−0.17	10	−0.40	0.0230
0.83	−0.08	15		0.0213
1.17	+0.07	22		0.0214
1.67	+0.22	28		0.0221
3.00	+0.48	36		0.0244
5.33	+0.73	42		0.0260
Average				0.0232

consistency of gelatin judged by partially inverting the test tube with its contents.

The results obtained with mixtures of gelatin with the same culture solution, in two separate determinations, are shown in table 1 and figure 1.

C was estimated as follows: T was plotted as ordinate and $\log t_s$ as abscissa as in figure 1. If K is a constant and T is zero, $\log t_s = C$. Hence project the "best" straight line through the experimental points to $T = 0$. Ideally, C should be the logarithm

of the time of setting of the gelatin mixture without enzyme. While such direct control furnishes values of C of the right order of magnitude, there is found an appreciable difference between C as determined graphically and as determined in the mixture. However, the graphically estimated C values were selected arbitrarily in the estimation of K.

Table 1 and figure 1 show that if the time of setting is very short, near that of the control, the error in judging the time of attainment of the selected consistency makes a larger proportional error in the true value of t_s.

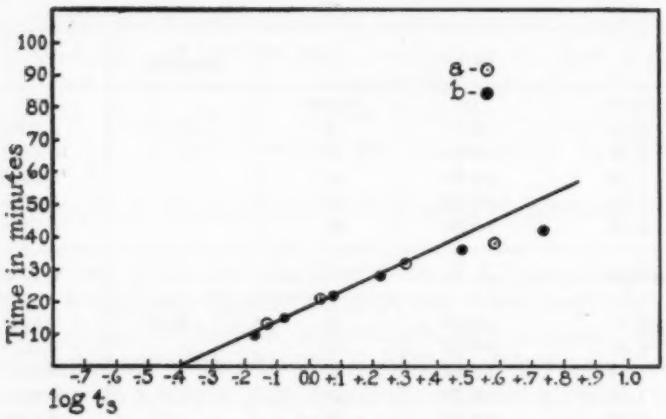

FIG. 1

On the other hand, if the time of setting is very large it means that extensive alteration of the gelatin has taken place and the equation found empirically to fit a short range of conditions would not be expected to hold. Hence the intermediate values of t_s in a series of measurements with different incubation time T were chosen as the more probable determinations of K. See table 1 and figure 1.

Effect of dilution on gelatinase activity

In order to determine the effect of dilution on gelatinase activity, mixtures of gelatin were made as previously described in

which varying amounts of phenolized bacterial culture solution were added to gelatin "solution" A, the final volumes of the

TABLE 2

K as a function of enzyme strength

Cubic centimeters phenolized enzyme solution mixed with 100 cc. phenolized gelatin "solution" A diluted to 125 cc.

CULTURE SOLUTION	K	$\frac{K}{s}$
cc.		
25	0.037	0.00148
20	0.029	0.00145
15	0.023	0.00153
10	0.015	0.00150
5	0.008	0.00160
2.7	0.004	0.00159
Average		0.00153

mixtures being kept constant by the addition of water and the final pH of the mixture for digestion with the enzyme controlled to pH 8.4 by previous additions of the proper amount of N

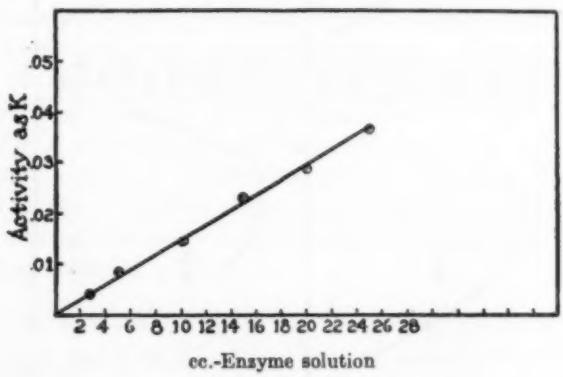

FIG. 2

sodium hydroxide solution. The test portions were all adjusted to pH 6.2 before cooling.

The effect of dilution of the gelatinase on the activity K is shown in table 2 and figure 2.

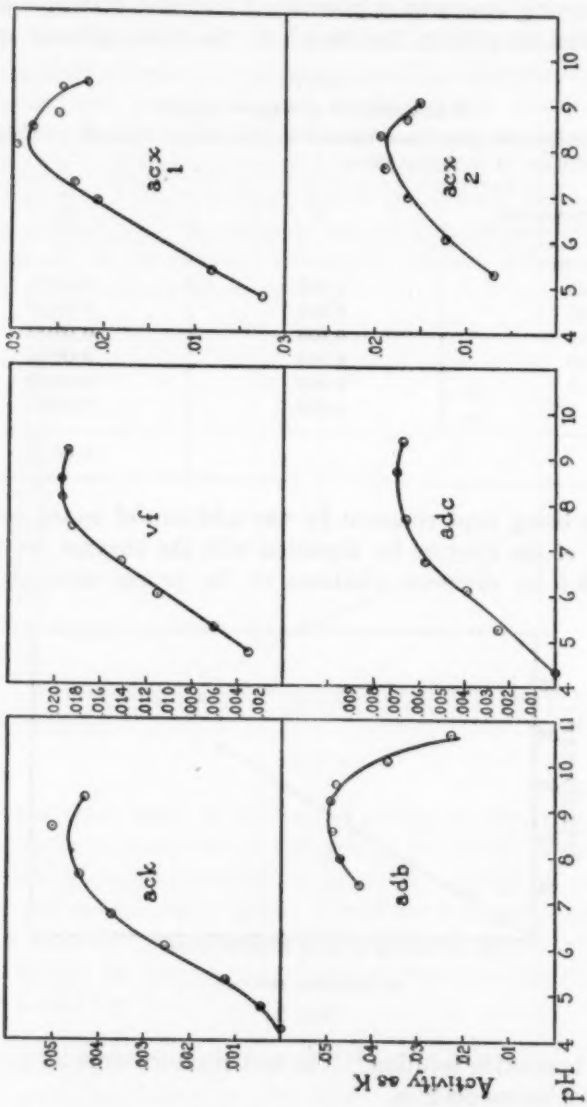

Fig. 3

From a study of table 2 and figure 2, it is seen that at the dilutions studied the activity K varies with the concentration of the gelatinase solutions. We therefore conclude that the activity K determined in cultures of the same strain of Proteus indicates the amount of gelatinase present in the culture solution.

Effect of pH of digestion mixture on gelatinase activity

The effect of hydrogen ion concentration on the rate of digestion of the gelatin by the enzyme produced in Proteus cultures, was determined by the method described above. One-half of 1 per cent phenol was added to a culture to prevent further bacterial action and digestion of gelatin determined at varying hydrogen ion concentrations. Before a test was made in ice water, the portion of gelatin-enzyme mixture removed was brought in these experiments, to pH 6.2.

In figure 3, the velocity constant is seen to be greatest in gelatin mixtures at a range near pH 8 to 9, and decreases on either side. At pH 4.7 the enzyme effect is small, a result which is in accord with those of Northrop (1922) who showed that trypsin digestion is at a minimum at the isoelectric point of gelatin.

Our results indicate the tryptase type of protease which Dernby (1921) found in his work on bacterial cultures. Within the limits of the proximate method we have found the optimum pH of activity of gelatinase in gelatin digestion to be about 8.4. Accordingly in subsequent experiments having to do with factors controlling gelatinase production, gelatin digestion was carried on near pH 8.4. Controls were run with gelatin at pH 8.4 without addition of gelatinase. No liquefaction was noted in these controls when chilled at pH 6.2.

Effect of pH of culture on production of gelatinase

Figures 4 and 5 illustrate experiments performed to determine the effect of the pH values during culture upon production of gelatinase.

The medium used was made as follows: 2 per cent Difco peptone, 0.5 per cent $Na_2HPO_4 \cdot 2H_2O$, 0.5 per cent KH_2PO_4, and 0.1 per cent NH_4Cl. Adjustments to the different initial pH

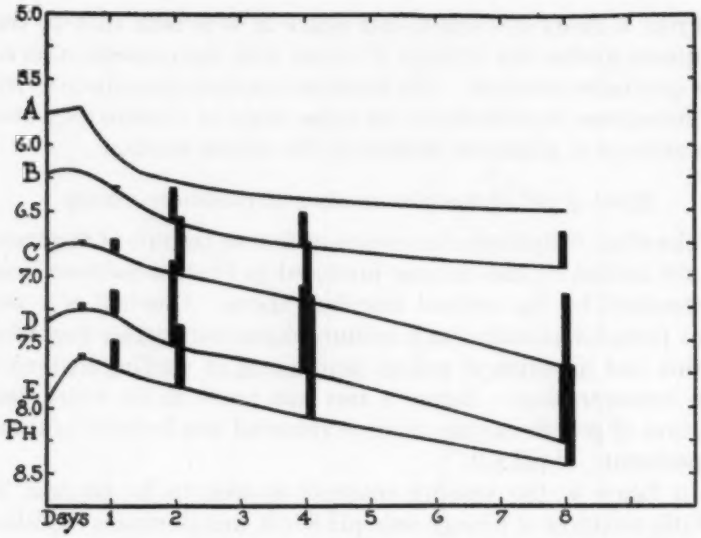
5.0
5.5
A
6.0
B
6.5
C
7.0
D
7.5
E
8.0
PH
8.5
Days 1 2 3 4 5 6 7 8

FIG. 4

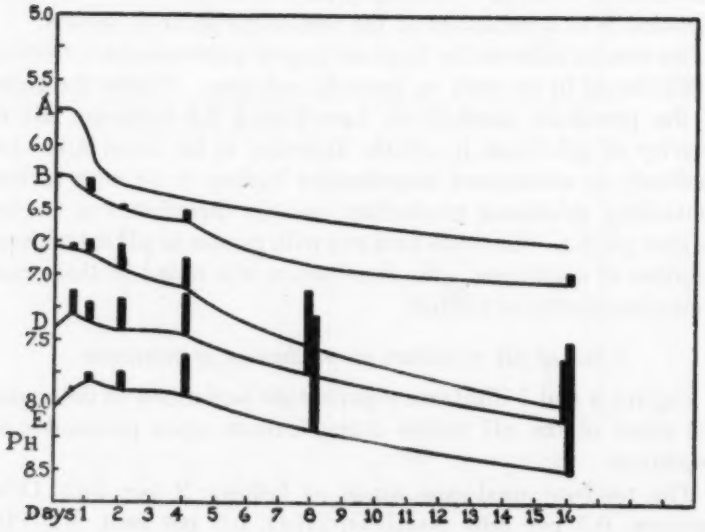
5.0
5.5
A
6.0
B
6.5
C
7.0
D
7.5
8.0
E
PH
8.5
Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIG. 5

values were made with hydrochloric acid or sodium hydroxide solution. The initial pH values were those measured after sterilization. An aliquot of each medium was placed in a 1000-cc. flask, inoculated with Proteus and incubated at 30°. Portions were withdrawn with sterile pipettes for the several tests.

In figures 4 and 5 the heights of the blacked rectangles represent the relative strengths of the enzyme produced as measured by the method outlined above. Each experiment was run in duplicate and the average value of each pair recorded.

The best production of gelatinase by Proteus in these experiments was found to be in cultures of reactions between pH 7 and 8. However, the data must be interpreted as applying to the particular conditions used. In cultures grown later in glucose media of original pH 7, we often obtained good production of gelatinase although the final culture reactions were quite acid.

Effect of aeration of culture on production of gelatinase

Liborius (1886) and others have noted the favorable effect of aeration on the production of proteolytic enzyme. The following experiments were made to study the effect of aeration on the production of gelatinase.

Proteus adc was grown in a medium of 2 per cent Difco peptone, 0.5 per cent $Na_2HPO_4 \cdot 2H_2O$ and 0.5 per cent KH_2PO_4 at a temperature of 30°. The conditions of aeration were as follows:

A_1 and A_2	Culture highly evacuated and sealed
B_1 and B_2	Culture in test tube with marble to delay diffusion
C_1 and C_2	Culture in open test tube
D_1 and D_2	50 cc. culture in 200 cc. Erlenmeyer
E_1 and E_2	50 cc. culture in 1 liter Erlenmeyer
F	Culture in tube with intermittent bubbling of O_2.

The production of gelatinase under these conditions is shown in table 3. The production of gelatinase as shown in this table evidently increases with increased aeration.

Since the aeration may affect the production of gelatinase by its effect on growth alone, an attempt was made to determine

the effect of aeration by contrasting cultures grown under apparently slight differences of air exposure.

For this experiment, a peptone-broth medium containing 1.0 per cent peptone, and 1.0 per cent $Na_2HPO_4 \cdot 2H_2O$, was adjusted to pH 7.2. Fifty cubic centimeters of the medium were placed in a 1000-cc. Erlenmeyer flask[6] and 500 cc. of the medium placed in another 1000-cc. Erlenmeyer flask and sterilized. The 50 cc. medium had a depth of 1.0 cm. and the 500 cc. medium had a depth of 4.4 cm., so that the aeration of the first was notably greater than that of the second. Both were inoculated with Proteus, strain acx, so that about 400 bacteria were added per

TABLE 3

Effect of aeration on production of gelatinase by Proteus

EXPERIMENT	*K* VALUES
A_1	0.0005
A_2	0.0004
B_1	0.0007
B_2	0.0009
C_1	0.008
C_2	0.009
D_1	0.027
D_2	0.027
E_1	0.064
E_2	0.043
F	0.050

cubic centimeter of media. The cultures were incubated at 30° and at intervals the number of bacteria per cubic centimeter was determined in each culture in order to determine the growth.

In table 4, the bacterial counts during growth are tabulated.

Table 4 shows that the growth of the bacteria is about the same in both cultures after sixteen hours. After that period there are more bacteria found per cubic centimeter in the shallow culture

[6] In all reference in this paper to cultures in a 1000 cc. Erlenmeyer flask, an Erlenmeyer flask of this volume is specified with a maximum diameter of approximately 13 cm. and a height of approximately 23 cm. with a cotton plug inserted in the neck, to allow aeration of the contents.

than in the deep culture. After fifty hours growth the activity of the gelatinase produced in the cultures was $K = 0.0207$ in the shallow culture and $K = 0.0015$ in the deep culture. In this experiment the greater aeration of the shallow culture resulted in a somewhat better growth of the bacteria, and production of a stronger gelatinase than was found in the culture of more depth and less aeration.

TABLE 4

Effect of aeration due to depth of culture on growth of Proteus

TIME OF GROWTH	NUMBER OF BACTERIA PER CUBIC CENTIMETER IN CULTURE OF DEPTH 1.0 CM.	NUMBER OF BACTERIA PER CUBIC CENTIMETER IN CULTURE OF DEPTH 4.4 CM.
hours		
1		
2		
3	$10^{2.3}$	$10^{2.0}$
4	$10^{3.5}$	$10^{3.5}$
5	$10^{4.0}$	$10^{4.3}$
6	$10^{4.6}$	$10^{4.6}$
7	$10^{5.0}$	$10^{4.9}$
8	$10^{5.9}$	$10^{6.3}$
9	$10^{6.6}$	$10^{6.6}$
10	$10^{7.3}$	$10^{7.3}$
11	$10^{7.7}$	$10^{7.9}$
12	$10^{8.3}$	$10^{8.0}$
13	$10^{8.3}$	$10^{8.6}$
14	$10^{8.3}$	$10^{8.3}$
15	$10^{8.6}$	$10^{8.5}$
16	$10^{8.7}$	$10^{8.6}$
17	$10^{8.5}$	$10^{8.3}$
19	$10^{9.0}$	$10^{8.3}$
21	$10^{8.9}$	$10^{8.3}$
23	$10^{9.3}$	$10^{8.6}$
42	$10^{10.0}$	$10^{9.3}$
52	$10^{10.0}$	$10^{9.0}$

The number of bacteria and gelatinase production was determined in two cultures of Proteus, strain acx, grown in a medium made of 2 per cent peptone, 2 per cent glucose, and 1 per cent $Na_2HPO_4 \cdot 2H_2O$, at a temperature of 30°, for seventy-two hours. The cultures were grown in 1000-cc. Erlenmeyer flasks, one in a 50 cc. portion of depth approximately 1 cm., the other in a 300 cc. portion with a depth of 3 cm.

In this experiment the small difference in numbers of bacteria in the cultures of different depth, does not account for the greater production of gelatinase in the more shallow culture which has the greater aeration, as shown in table 5.

In order to study the effect of aeration still further, another experiment was carried out in which the aeration was more definitely controlled. Two cultures of Proteus strain acx were grown in peptone-broth containing 1.0 per cent $Na_2HPO_4 \cdot 2H_2O$. One culture was grown in 50 cc. of medium in a 1000-cc. Erlenmeyer flask providing good aeration. The other was grown in

TABLE 5

Effect of varying depth of culture on growth and production of gelatinase

DEPTH	FINAL NUMBER OF BACTERIA PER CUBIC CENTIMETER	K
cm.		
1	$10^{9.6}$	0.0168
3	$10^{9.4}$	0.0037

TABLE 6

Effect of aeration on growth and production of gelatinase by Proteus

CULTURE IN	FINAL NUMBER OF BACTERIA PER CUBIC CENTIMETER	GELATINASE ACTIVITY AS K	FINAL pH OF CULTURE
50 cc. open to aeration	$10^{9.4}$	0.0316	8.4
1000 cc. closed to aeration	$10^{9.6}$	0.0002	6.9

1000 cc. of medium held in a 1000-cc. Florence flask from which air was excluded by means of a rubber stopper with an outlet tube dipping into mercury to allow escape of evolved gases. The number of bacteria per cubic centimeter at the beginning of growth was about 300, and the original pH of the medium was pH = 7.2.

In table 6 are given the number of bacteria per cubic centimeter and the activity of the gelatinase determined in the culture after ninety-six hours incubation at 30°. From the results in this table, it appears that the small difference in growth of the bacteria

in the two cultures does not account for the much greater production of gelatinase in the culture which is well aerated. The difference in pH of the culture is not the reason for the difference in production of gelatinase, as, in many cultures previously studied, a good production of gelatinase was found in aerated cultures at pH 6.9.

From the experiments on the effects of aeration on the production of gelatinase by Proteus, it appears that access of air to the culture results in stimulation of production of gelatinase due to aerobic effects, in themselves not associated with numbers of bacteria or with hydrogen ion concentration, since the number of bacteria under anaerobic conditions may be near that of aerobic cultures, and the difference in hydrogen ion concentration does not account for so large a difference in production of gelatinase.

A further discussion of the effect of aeration on production of gelatinase by Proteus will be given in the section on synthetic media.

REPRODUCIBILITY OF RESULTS

While very fair duplications of data were obtained in parallel experiments made at the same time, with the same materials and with the same culture, it was difficult to reproduce the data with experiments made at other times and with different samples of peptone in the medium.

Under apparently the same conditions a variation of the brand of peptone used causes a wide variation in gelatinase production and it was realized that other factors beside pH and aeration had still to be considered. We therefore began the study and development of synthetic media of well controlled composition which might take the place of the peptone-broth in the growth of Proteus with production of gelatinase.

SYNTHETIC MEDIUM DEVELOPED FOR CULTURE OF PROTEUS WITH PRODUCTION OF GELATINASE

It has been shown by Brunton and Macfadyen (1889), Fermi (1892), Jordan (1906), Diehl (1919) and others that the same bacteria which produce proteolytic enzyme in one kind of a

medium will not produce it in another medium, although the growth may be luxuriant in both.

Fermi, who found good enzyme production in gelatin cultures of certain species of bacteria, also grew a number of bacteria in synthetic media in which ammonium salts were the only source of nitrogen. A trace of proteolytic enzyme was found in the cultures of but three species of bacteria out of the number studied and he concluded that little or no proteolytic enzyme could be produced by bacteria in protein-free nutrient media.

Jordan used a synthetic medium in which asparagin was the source of nitrogen. He obtained enzyme but his tests were made on cultures many weeks old.

Diehl obtained good enzyme in synthetic media with three species of bacteria when asparagin was present as a source of nitrogen but none when ammonium salts were the only form of nitrogen. He concluded that organic nitrogen was necessary for the production of proteolytic enzyme.

Auerbach (1897) obtained a better production of proteolytic enzyme in bacterial cultures when he added calcium or magnesium carbonate to the peptone broth media. He ascribed this effect to the neutralizing action of these salts. In our work we have shown that the effect of calcium or magnesium salts is due to some other factor than the pH of the culture.

Soppeland and Levine (1924) found that calcium and sodium chloride together accelerate the proteolysis of milk by certain strains of bacteria. They did not show whether this was due to an accelerating effect on the action of the enzyme on its substrate, or to a greater production of proteolytic enzyme. Levine and Soppeland (1926) studied the effect of sodium chloride and calcium chloride on proteolysis of gelatin by bacteria but here again, the bacteria were grown in the gelatin medium, and one cannot separate the effects of the salts on the production of enzyme by the bacteria from the effects on the liquefaction of the gelatin by the enzyme. Delezenne (1905) and Delezenne and Zunz (1906) have shown that calcium salts may accelerate proteolysis by pancreatic extract.

Falk (1923), in a review of synthetic media, has shown that Ca

is often left out of the composition of synthetic media while Mg is frequently retained.

In our work we have found that calcium and magnesium salts have considerable influence on the production of proteolytic enzyme by bacteria and that this enzyme may be produced by Proteus when ammonium salts are the only source of nitrogen if calcium or magnesium salts be present.

The first synthetic medium was made as follows:

Synthetic medium no. 1

$Na_2HPO_4 \cdot 2H_2O$*	8.8 grams
K H phthalate	2.0 grams
Asparagine	1.0 gram
Glucose	1.0 gram
Glycerol	1.0 gram
Distilled H_2O	to 1000 cc.

Adjusted to pH 7.0 with N/1 HCl.

* The $Na_2HPO_4 \cdot 2H_2O$ was added as usual in the synthetic media in part for its action as a buffer salt in the culture.

Fifty-cubic-centimeter portions of synthetic medium no. 1 were placed in 1000-cc. Erlenmeyer flasks under aseptic conditions. Inoculation was made with Proteus strain acx so that 10^3 bacteria were added per cubic centimeter medium. After five days incubation at 30° the bacteria had increased to $10^{9.0}$ per cubic centimeter. No gelatinase was found in this medium. Transfers were made of this culture to fresh synthetic medium several times with the same result. Growth was slow but the same number of bacteria were finally obtained as in peptone media.

Next a more concentrated synthetic medium was made of different composition.

Synthetic medium no. 2

Na_2HPO_4	10 grams
KH_2PO_4	2 grams
Asparagine	10 grams
Glucose	10 grams
Distilled H_2O	to 1000 cc.

Adjusted to pH 7.0 with N/1 HCl.

When 0.2 per cent cystine or 0.2 per cent sodium thiosulfate was added to this medium, growth was obtained but no gelatinase.

A synthetic medium no. 3 was made up as follows with ammonium salts as sole source of nitrogen.

Synthetic medium no. 3

$Na_2HPO_4 \cdot 2H_2O$	10 grams
KH_2PO_4	2 grams
NH_4Cl	10 grams
Glucose	10 grams
Distilled H_2O	to 1000 cc.

Adjusted to pH 7.0.

When Proteus strain acx, was transferred to 50 cc. of synthetic medium no. 3 in a 1000-cc. Erlenmeyer flask, fair growth of the bacteria was obtained but no production of gelatinase. However, when 0.05 per cent calcium carbonate and 0.05 per cent magnesium sulfate were added to 50 cc. medium no. 3 and other cultural conditions repeated, Proteus strain acx grew to the same maximum numbers as in peptone broth and a good production of gelatinase was obtained.

Preparation of synthetic media

Precautions were taken never to sterilize the ammonium chloride and sugar solutions together with heat. The ammonium chloride solution and the phosphate solution were each sterilized separately at 15-pound pressure in the autoclave. When heated together free ammonia was given off. The sugar solution was passed through a Berkefeld filter to make it sterile. Care was taken not to pass ammonium or phosphate solutions through the Berkefeld as calcium and magnesium salt impurities might well be extracted from the filter under these conditions. The Berkefeld filter was well washed with distilled water before use to remove soluble salts developed when sterilized by heat. The solutions were mixed in the proper proportions after sterilizing as above.

All work with bacteria was done under aseptic conditions.

Salts and sugar were recrystallized twice except for the calcium and magnesium salts which were high grade analyzed salts used

in small quantities. All glassware was made specially clean before use. Flasks used for cultures were of Pyrex glass.

Proteus acx[7] was adapted to the synthetic medium used, and after growth diluted to 10^7 and 1 cc. added to fresh synthetic medium so that from 100 to 1000 bacteria were added per cubic centimeter of medium. Transfers were made several times so that growth effects were known to be due to the synthetic medium without traces of any peptone. Fifty cubic centimeters of the synthetic medium were added to 1000-cc. Erlenmeyer flasks to insure surface aeration of shallow cultures. Calcium and magnesium salts in weighed amounts were sterilized in the flasks previous to addition of the solutions.

The strength of the gelatinase produced in synthetic medium cultures was not as great as that produced in peptone broth cultures. Nevertheless on mixing the cultures with gelatin in the same proportion as used in previous determinations of gelatinase, it was found that the best gelatinase production in synthetic media resulted in liquefaction of the gelatin in from two and one-half to three hours digestion time. This production of gelatinase is called "good" in the tables on results obtained in synthetic medium.

When the production of gelatinase was such that it took twenty-four hours digestion with the gelatin to obtain liquefaction, the gelatinase is called "fair." When only a slight liquefaction took place after three days digestion, the enzyme is designated "small."

Effect of calcium and magnesium salts

Since a "good" production of gelatinase was obtained when Proteus was grown in synthetic medium no. 3 to which both calcium carbonate and magnesium sulfate had been added, the effect of these salts was studied in a series of experiments conducted, in the main, as already described. In those cultures in which no magnesium sulfate was present 0.025 gram sodium

[7] In all work with the synthetic media Proteus strain acx was used. The results recorded apply to strain acx. The effects of the synthetic media on other strains of Proteus have not yet been investigated.

sulfate was added so that the sulfate ion might be present in the control. Twenty-five-thousandths gram sodium carbonate was added to those cultures in which no calcium carbonate was present to insure the carbonate ion. The initial pH of all cultures was 7.0 to 7.1.

In tables 7 and 8 the effects of 0.05 per cent calcium and magnesium salts are shown on the growth and production of gelatinase by Proteus grown in 50 cc. synthetic medium no. 3 in 1000 cc. Erlenmeyer flasks under aerobic conditions. Inoculations were

TABLE 7

The effect of calcium and magnesium salts on the growth and production of gelatinase by Proteus in synthetic medium no. 3

ADDITION OF Ca AND Mg	NUMBER OF BACTERIA PER CUBIC CENTIMETER AFTER						PRODUCTION OF ENZYME AFTER 4 TO 10 DAYS GROWTH
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	
grams							
0.025 $CaCO_3$ 0.025 $MgSO_4$	10^{8}	$10^{8.5}$	$10^{8.6}$	$10^{9.6}$	$10^{9.6}$	$10^{9.7}$	"Good"
0.000 $CaCO_3$ 0.000 $MgSO_4$ 0.025 Na_2SO_4	$10^{2.3}$	$10^{4.8}$	$10^{7.9}$	$10^{8.8}$	$10^{8.5}$	$10^{8.6}$	"None"
0.025 $CaCO_3$ 0.000 $MgSO_4$ 0.025 Na_2SO_4	$10^{1.0}$	$10^{4.1}$	$10^{7.3}$	$10^{8.7}$	$10^{8.8}$	$10^{8.7}$	"Fair"

made so that about 100 bacteria were added per cubic centimeter medium.

For the determination of gelatinase 2.5 cc. of culture was added to 10 cc. of gelatin "solution" A, the pH of which was such that on mixing with the culture the pH of the mixture would be 8.4. The mixtures were kept in test tubes in a water bath at 30° and portions removed as usual for tests.

The pH of these cultures, after several days' growth, was 6.8 to 7.0 so that the differences in the results were not due to variation of the hydrogen ion concentration of the culture.

Gelatinase was found in the culture containing both calcium

and magnesium on the fourth day of growth when the growth had attained the maximum value. This enzyme increased in strength up to the seventh day of growth.

In the culture containing calcium but no magnesium, a weak gelatinase was found on the fourth day of growth. This enzyme increased in strength at a later period but did not attain the strength of the gelatinase in the first culture containing both calcium and magnesium.

TABLE 8

The effect of calcium and magnesium salts on the growth and production of gelatinase by Proteus in synthetic medium no. 3

Addition of Ca and Mg	Number of bacteria per cubic centimeter after						Production of enzyme after 4 to 10 days' growth
	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	
grams							
0.025 $CaCO_3$ 0.025 $MgSO_4$	$10^{2.6}$	$10^{5.1}$	$10^{8.0}$	$10^{9.5}$	$10^{9.8}$	$10^{9.7}$	"Good"
0.000 $CaCO_3$ 0.000 $MgSO_4$ 0.000 Na_2SO_4*	$10^{2.7}$	$10^{5.5}$	$10^{8.2}$	$10^{8.5}$	$10^{8.8}$	$10^{8.7}$	"Trace"
0.000 $CaCO_3$ 0.025 $MgSO_4$ 0.025 Na_2CO_3	$10^{2.7}$	$10^{5.7}$	$10^{8.5}$	$10^{9.4}$	$10^{9.7}$	$10^{9.8}$	"Small"
0.025 $CaCO_3$ 0.025 $MgSO_4$ 0.025 Na_2CO_3	$10^{2.6}$	$10^{4.8}$	$10^{8.2}$	$10^{9.2}$	$10^{9.6}$	$10^{9.7}$	"Good"

*Sodium sulfate was added to the control in the previous series without apparent effect.

No enzyme was found at all in the culture in which there was no calcium or magnesium.

In this series (table 8) gelatinase was found in the culture containing calcium and magnesium after four days when the number of bacteria had reached maximum values. In the culture containing magnesium and no calcium only a slight production of gelatinase was found after eight days' growth. The number of bacteria determined was practically the same in both

these cultures and the difference in production of gelatinase is not due to any significant difference in growth of these two cultures.

Only a trace of gelatinase was found in the culture containing no calcium and no magnesium. Although the original pH of the cultures was 7.0 to 7.1 the pH of the cultures in the second series, after eight days incubation, was 6.5 to 6.4. This hydrogen ion concentration was not the cause of the difference in enzyme production as both comparatively good and poor production of gelatinase occurred at this pH of the series. It is apparent that the addition of calcium and magnesium salts results in production of gelatinase, which is best when both salts were added, although with calcium alone a fair production of gelatinase is obtained. The difference in production of enzyme was not due to the effect of the anions of the salts, as they were added in the controls as sodium sulfate or sodium carbonate.

The smaller numbers of bacteria do not seem to be the cause of the apparent *lack* of enzyme in the cultures without calcium or magnesium salts, as digestion with gelatin was prolonged three days within which time a relatively small enzyme would have given liquefaction had it been present.

When calcium sulfate was substituted for the carbonate a good production of gelatinase was obtained in these cultures.

An experiment was made which showed that the differences found in the gelatinase activity of cultures were due to calcium and magnesium salt effects in the cultures and not to salt effects on the digestion of the gelatin by the enzyme. A series of cultures was made in synthetic medium as in the previous experiments, with and without the addition of calcium and magnesium salts. After several days growth the gelatinase was tested as usual. Two-and-five-tenths cubic centimeters of each culture solution was added to 10 cc. portions of gelatin "solution" A in test tubes. At the same time 2.5 cc. of each culture solution was added to 10 cc. portions of gelatin "solution" A to which had been added a solution of calcium or magnesium salts, so that each gelatin mixture contained the calcium or magnesium salt lacking in the culture added to it. Care was taken to keep the volumes

and pH of these mixtures alike. It was of interest to find that in this experiment the velocity of digestion of the gelatin by the gelatinase in the cultures was unaffected by the absence or presence of the calcium or magnesium salts in the gelatin digestion mixture.

The culture solution lacking both calcium and magnesium salts did not liquefy the gelatin mixture to which calcium and magnesium salts were added. The culture solution containing magnesium salt but no calcium salt showed the same "small" gelatinase activity in the gelatin mixture with and without addition of calcium salt; and the culture solutions containing calcium and no magnesium, showed the same "fair" gelatinase activity in the gelatin mixture to which magnesium salt had been added as in its control. It may be concluded then that the salt effects found in the cultures grown in our synthetic medium were due to effects on production of gelatinase in the cultures themselves.

It was thought that possibly the salt effects might be due to autolysis of the bacterial cells. Phenol was added to cultures containing no calcium or magnesium during growth and these killed bacterial mixtures were added to calcium and magnesium salts and allowed to stand several weeks. No indication of enzyme due to autolysis was found.

Further modification of the synthetic medium

It was found that when Proteus was grown in synthetic media in which the amount of glucose and ammonium chlori le was reduced to 0.5 per cent, a final pH of 6.8 resulted when calcium and magnesium were present; while in a medium which contained larger amounts of glucose and ammonium chloride a tendency was noted for the final pH of the culture to be lower. A somewhat stronger enzyme was obtained in the cultures grown in the modified synthetic media in which the final reaction was pH 6.8. In this experiment 50 cc. of synthetic media in 1000-cc. Erlenmeyer flasks were inoculated as usual and the results are shown in table 9.

A further change was then made. Lactic acid, neutralized with N sodium hydroxide solution[7] was substituted for glucose in

the synthetic media; with calcium and magnesium salts added this modified medium gave good growth of Proteus and "good" production of gelatinase. The culture became alkaline as the organic acid was decomposed by the bacteria. The effect of

TABLE 9

Effect of decreasing ammonium chloride and glucose in the synthetic medium on bacterial numbers, pH and enzyme activity K

AMMONIUM CHLORIDE	GLUCOSE	FINAL NUMBER OF BACTERIA PER CUBIC CENTIMETER	FINAL pH OF CULTURE	$100 \times K$
per cent	*per cent*			
0.5	0.5	$10^{9.8}$	6.8	0.393
1.0	0.5	10^{10}	6.8	0.333
1.0	1.0	$10^{9.8}$	6.2	0.192

TABLE 10

The effect of calcium and magnesium salts on the final number of bacteria, pH and production of gelatinase by Proteus in lactate synthetic medium

NUMBER	Ca AND Mg	FINAL NUMBER OF BACTERIA PER CUBIC CENTIMETER	FINAL pH OF CULTURE	PRODUCTION OF ENZYME
	grams			
1	0.043 $CaSO_4 \cdot 2H_2O$ 0.025 $MgSO_4$	$10^{9.0}$	7.6	"Good"
2	0.043 $CaSO_4 \cdot 2H_2O$ 0.000 $MgSO_4$	$10^{9.3}$	7.2	"Fair"
3	0.000 $CaSO_4 \cdot 2H_2O$ 0.025 $MgSO_4$	$10^{9.3}$	7.7	"Trace"
4	0.000 $CaSO_4 \cdot 2H_2O$ 0.000 $MgSO_4$ 0.025 Na_2SO_4	$10^{8.6}$	7.0	"None"

calcium and magnesium salts was studied in a series in which 0.5 per cent lactic acid was substituted for glucose in the synthetic medium and 0.5 per cent ammonium chloride was used instead of 1 per cent. Calcium was added as calcium sulfate and in amount equivalent to that used in the first and second series.

The cultures were grown in 50 cc. shallow layers in 1000-cc. Erlenmeyer flasks as in the previous series and the initial pH was 7.0 to 7.1.

Table 10 records the findings at the end of four days' growth. In this series, the growth is the same for the first three cultures and somewhat less in the culture in which no calcium or magnesium is present.[8] The lack of enzyme in no. 3 in which magnesium is present but no calcium is not due to any decrease in numbers of bacteria since the growth is as good as in 1 and 2.

In no. 4, in which no calcium or magnesium is present, the number of bacteria, although somewhat less than in the other cultures, cannot account for the fact that no enzyme was produced as digestion with gelatine was prolonged sufficiently long to give indication of any enzyme present.

The final hydrogen ion concentration of the cultures is not the cause, as at pH 7.6 and 7.7, a good enzyme is produced with calcium and magnesium together and a poor one with magnesium alone. At pH 7.2 and 7.0, a fair enzyme is produced with calcium alone and no enzyme is produced in the culture without either calcium or magnesium.

It appears again that calcium and magnesium together give the strongest enzyme; that calcium alone gives a fair enzyme and that magnesium alone gives a trace of enzyme, while in cultures with neither calcium nor magnesium, no determinable enzyme is produced during the period studied.

After ten days' growth, the alkalinity increased in cultures nos. 1, 2 and 3 to a pH of 8.1 to 8.4. The increasing alkalinity had resulted in a slight decrease in numbers of bacteria from $10^{9.3}$ to $10^{8.5}$ and the enzyme produced had been partially destroyed.

No enzyme was found in the lactate culture without calcium or magnesium, even *after twenty-eight days'* growth.

[8] The error in determination of bacterial numbers is not small, but from consideration of the numbers of bacteria found in cultures grown in the synthetic medium referred to in this paper, one may conclude that the production of gelatinase may be small in spite of large numbers of bacteria. In cultures containing magnesium and no calcium, the number of bacteria was as great as in cultures containing calcium, but in the former case, very little gelatinase was produced compared to the latter.

The amounts of calcium and magnesium salts used in the above experiments were more or less arbitrary and usually resulted in the presence of a solid phase.

A series of Proteus cultures was grown in 50 cc. portions of media containing decreasing amounts of calcium and magnesium salts in the lactate synthetic medium. The cultures were grown in 1000 cc. flasks as before. It was found that "fair" production of gelatinase was obtained in those cultures containing calcium chloride equivalent to as little as 0.0025 per cent $CaCO_3$, and no magnesium salt. No solid phase occurred when 0.005 per cent Ca salt was added alone.

The fact that so little as 0.0025 per cent calcium salts in cultures grown in synthetic medium resulted in a "fair" production of gelatinase, while without calcium salt in the control culture no gelatinase was found, impresses the investigator with the importance of careful cleansing of glassware used in work of this character.

Only a "small" production of gelatinase took place in those cultures containing magnesium sulfate alone, although the strength of the enzyme was the same in those cultures containing 0.0024 per cent magnesium sulfate as in those of 0.05 per cent magnesium salt content. When 0.012 per cent calcium and 0.012 per cent magnesium salts were both present a "good" production of gelatinase was obtained.

With neither calcium nor magnesium salts, no gelatinase was produced in the synthetic medium cultures during the period of observation.

EXPERIMENTS ON ANAEROBIC GROWTH AND PRODUCTION OF GELATINASE BY PROTEUS IN SYNTHETIC MEDIA

Anaerobic cultures of Proteus were grown in 1-liter Florence flasks, filled to the neck with synthetic media. A rubber stopper was inserted, through which two glass tubes allowed nitrogen to bubble until little or no air was left in the flask. This nitrogen had been deoxygenated by passage over reduced copper at 700°. The inlet tube was then closed, the bent end of the outlet passing into mercury which allowed any evolved gases to escape while no air could enter.

In the first series 0.5 per cent lactic acid (neutralized with sodium hydroxide) was substituted for glucose in the synthetic media. Calcium and magnesium salts were present 0.025 per cent each.

In one flask, sodium nitrate was added to make a concentration of 0.25 per cent.

After ten days' incubation, it was found that the bacterial numbers had increased from $10^{2.8}$ to $10^{7.2}$ per cubic centimeter in the culture to which nitrate had been added, while in the cultures containing no nitrate, there was an increase to only $10^{4.0}$ bacteria per cubic centimeter. Also, a fairly strong gelatinase was found in the culture containing nitrate but none in the culture without it. The final pH of both was about 7.0.

Quastel and Stephenson (1925) have shown that Proteus is able to grow anaerobically in their synthetic media. Here, lactic acid was the organic carbon compound, and nitrate was present. Quastel, Stephenson and Whetham (1925) found that certain bacteria ceased to grow when 0.4 per cent nitrite was formed by reduction.

In our experiment fair growth was obtained when only 0.25 per cent nitrate was added. We have shown that a fairly strong gelatinase is produced under these anaerobic conditions with the number of bacteria somewhat below the maximum number, while without nitrate, no enzyme is found.

Another series was then run under anaerobic conditions in which the synthetic medium contained glucose instead of lactate, and calcium and magnesium were added. The bacterial numbers increased to $10^{6.6}$ per cubic centimeter in the culture to which no nitrate was added and a fairly strong gelatinase was found, which shows that glucose without nitrate not only supports anaerobic growth of Proteus but also allows a small production of gelatinase in this synthetic medium under anaerobic conditions.

In a culture grown as above, but with magnesium and no calcium present, no enzyme was found, showing again the more favorable influence of calcium as previously determined under aerobic conditions.

Dernby and Blanc (1921) obtained a proteolytic enzyme in cul-

tures of anaerobes grown in broth to which 1 per cent glucose had been added and a "minimal" dose of calcium sulfide. Our results differ in that a facultative anaerobe was used instead of the obligate spore-forming anaerobe used by Dernby and Blanc, and that our cultures were grown in synthetic media instead of in broth.

The importance of the composition of the medium in which bacteria are grown for the study of the effect of anaerobiosis on the production of gelatinase is shown in our results obtained with synthetic media.

DISCUSSION

The importance of using a synthetic medium in place of peptone-broth for growth of bacteria in investigations dealing with production of gelatinase by bacteria, has been shown in our present work. Synthetic media such as those described in this paper, can be easily duplicated while the composition of peptone-broth varies with the peptone used. We have been able to duplicate our results a number of times in synthetic media but results with peptone-broth are not uniform.

It is of considerable interest that the presence of calcium or magnesium salts in the synthetic media should have such an effect on the production of gelatinase by Proteus. This effect is not due to numbers of bacteria in the cultures as good growth was obtained in presence of magnesium salts with only "small" production of gelatinase, while "fair" or "good" production of gelatinase was obtained in presence of calcium.

Without either calcium or magnesium, the small decrease in growth of the bacteria which took place does not explain the apparent lack of gelatinase produced.

We have shown that the calcium and magnesium salt effects were not due to an effect on the liquefaction of gelatin by gelatinase but on the production of gelatinase in the culture solutions containing the salts.

The reason for the calcium and magnesium effects on production of gelatinase by Proteus is not apparent. It may be due to specific effects on the bacteria during growth. The antagonistic

action of salts is often discussed in regard to results with salts in bacteriological media, but we have no evidence on this point.

In regard to studies of the anaerobic effects on the production of gelatinase by bacteria, we have shown that the composition of the media is of great importance, since Proteus can produce gelatinase under anaerobic conditions, when glucose is present or when small amounts of nitrate are added to a lactate synthetic medium.

SUMMARY

The method for estimation of gelatinase as modified by Clark has been described in detail.

The effects of pH and aeration on production of gelatinase in cultures of Proteus have been studied.

The development of a synthetic medium in which Proteus gives good growth and production of gelatinase has been presented.

The effect of calcium and magnesium salts on the production of gelatinase has been studied and discussed.

The production of gelatinase by Proteus under anaerobic conditions has been successfully obtained in certain synthetic media.

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BACTERIAL METABOLISM

THE INFLUENCE OF PHOSPHATE BUFFER IN CARBOHYDRATE-FREE AND IN GLUCOSE-CONTAINING MEDIA

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Many attempts have been made to follow the metabolic changes brought about by bacteria in mixed and in pure culture in various kinds of media, but the complexity of the many deep-seated changes which take place in the organic substrates is such that it may be said but little progress has been made in this field of biochemistry.

Much attention has been directed in more recent years to a study of the immediate fate of complex nitrogenous substances, as revealed by ammonia production and by changes in amino acid content of the medium, and of the ability of microörganisms to destroy various carbohydrates, higher alcohols, etc., with the resultant production of acids and other products characteristic of such decomposition.

A subject of particular interest to investigators has been the influence of the presence of utilizable carbohydrates on the course of nitrogen metabolism, with not a little difference of opinion as to the real significance and explanation of altered metabolism brought about by such agents.

As early as 1886 Hirschler observed that the production of indol, phenol and cresols is inhibited by the presence of available carbohydrate.

Péré (1892) showed that, although sugar has a restraining effect on indol formation, the indol-producing function is not permanently lost. Inoculation from a fifteenth generation glucose broth culture into plain peptone resulted in the restoration

of the original proteolytic property of the organism. Péré claimed further (1893) that the relative amount of acid formed by the colon bacillus decreases with greatly increased amounts of peptone in the medium.

Peckham (1897) concluded that when members of the colon group are cultured under conditions favorable to the development of both proteolysis and fermentation, the latter invariably takes precedence, and that there is no evidence of proteolysis until fermentation has ceased.

De Graaf (1909) noted that glucose exerts an inhibitory effect on indol production. Rougentzoff (1913) reported that glucose, fructose, lactose and mannitol inhibit indol formation by *Bact. coli*. Indol was formed, however, in media containing sucrose, maltose and dulcitol.

Distaso (1913) was of the belief that in a test medium containing utilizable carbohydrate and tryptophan as the source of indol, the more readily available substances are used first by *Bact. coli*, and that consequently indol is not formed in ordinary sugar media. The above observations have been on the whole confirmed by numerous investigators.

Kendall, Day and Walker (1913) conducted a series of studies on the relative constancy of ammonia production by *Bact coli* and *Bact. typhosum*. They observed very little or no increase in ammonia in peptone broth containing glucose, whereas ammonia production in carbohydrate-free broth could be readily demonstrated. These and similar observations with other organisms led Kendall to maintain that fermentation takes precedence over putrefaction, and that glucose exerts a sparing action on protein when both are present. In their later work, Kendall and his associates extended their quantitative studies to amino nitrogen, total nitrogen and non-protein nitrogen, along with the ammonia determinations.

Liborius (1886) found that the presence of sugar did not inhibit gelatin liquefaction in cultures of *B. subtilis*, *Ps. fluorescens* and *Ps. pyocyanea*.

Sears (1915) observed that generally the concentration of free ammonia and amino acids was lower in glucose than in plain broth.

B. subtilis and *Vibrio metchnikovi* proved to be exceptions. *Cl. Welchii*, on the other hand, produced more ammonia and amino acids in the presence of glucose than in its absence.

Glenn (1911) concluded that indol production by *Bact. coli* and *Proteus vulgaris* is inhibited in sugar-containing media by the acid formed, and not by the carbohydrate *per se*.

Waksman (1920) held that a definite amount of ammonia may always be produced out of complex nitrogenous compounds, whether available carbohydrate is present or not, but that the ammonia may be utilized in the presence of the sugar, while in a sugar-free medium proteolytic organisms utilize the "protein" substances as a source of carbon as well as of nitrogen, leaving large quantities of ammonia in the medium.

Henderson and Webster (1907) found that the presence of phosphate greatly favors the growth of *Bact. acidi-lactici* (coli). They explained this function by the ability of phosphates to neutralize the acid, with the reservation that their observations did not conclusively establish this claim.

Fischer (1915) held that acidity plays no rôle in the inhibition of indol formation in sugar-containing media. Kligler (1915) maintained that while acid does inhibit liquefying enzymes, it is usually the sugar that is directly responsible for the absence of liquefaction. Jones (1916) attributed the failure of the enzymes of *Proteus vulgaris* to develop in sugar-containing medium to a paralyzing action of hydrogen ions on the endo-enzyme.

Kligler (1916) found that with moderate amounts of glucose present, ammonia production increased with increased concentration of peptone. He suggested that the greater ammonia production may have been due to the favorable buffer effect of increasing amounts of peptone.

Berman and Rettger (1918) observed that the presence of sufficient buffer (phosphate) in glucose-containing medium encourages continued normal nitrogen metabolism of *Proteus vulgaris*. The phosphate also tended to favor normal nitrogen metabolism of *Bact. coli* in the presence of relatively small amounts of glucose.

B. subtilis, though a glucose-utilizing organism, was able to

maintain its proteolytic activity in the presence of this sugar, without added buffer. This continued metabolic function was explained by Berman and Rettger by the assumption that the nitrogenous substances are attacked by this organism relatively easily, and that the organism is thus able to produce its proteolytic enzyme and consequently its own buffer to offset the free H ions formed from the glucose. They concluded that moderate amounts of utilizable carbohydrate do not affect the nitrogen metabolism of bacteria, provided favorable experimental conditions are maintained. The more luxuriant growth of organisms in glucose phosphate medium was explained on the ground that the nitrogenous constituents of the medium are utilized for structural purposes for which the glucose furnishes much of the energy supply, whereas in the plain broth the nitrogenous substrate furnishes both the structural and functional food requirements.

Bronfenbrenner and Schlesinger (1918) made a study of the metabolism of *Bact. coli* in peptone-phosphate-lactose water, and concluded that careful consideration should be given to the relation of buffer salts to carbohydrates in the conducting of fermentation experiments.

De Bord (1923) carried on metabolism studies in buffered (phosphate) medium. His results indicated that the production of amino acids was always greater in glucose than in plain broth, but that ammonia production was much greater in the sugar-free than in the glucose broth. He believed, as did Raistrick and Clark (1921), that free ammonia is not a reliable index of bacterial proteolysis.

EXPERIMENTAL

The main purposes of this investigation were to determine the influence of balanced phosphate on the course of nitrogen metabolism in glucose-containing and in carbohydrate-free peptone media, and to make a comparative study of the metabolic activities of certain selected organisms representing widely different types and groups.

The work involved a systematic quantitative determination of

total, protein, non-protein, ammonia, amino, and polypeptid nitrogen, and biuret and glucose. Also H ion concentration and turbidity.

The following 22 organisms were employed: *B. anthracis*, *B. subtilis* (2 strains), *B. cereus*, *B. megatherium*, *Bact. coli* (2 strains), *Bact. typhosum* (2 strains), *Bact. paratyphosum* A (2 strains), *Bact. paratypohsum* B (2 strains), *Bact. pullorum* (2 strains), *Proteus vulgaris*, *Bact. bronchisepticum*, *Ps. pyocyanea*, *E. prodigiosus*, *Staph. aureus*, *Cl. sporogenes*, *Cl. welchii*.

A solution of 1 per cent Witte peptone was used as the basic medium. This was employed as

(1) Plain peptone without glucose
(2) Plain peptone plus 1 per cent glucose
(3) Plain peptone plus phosphate buffer, without glucose
(4) Plain peptone plus phosphate buffer and 1 per cent glucose

In a few instances ammonium sulphate was also used to determine the fate of the added ammonia in the different media.

The buffer solution was made by dissolving 12 grams of K_2HPO_4 and 5 grams of NaH_2PO_4 in 200 cc. of distilled water. This mixture of the primary and secondary phosphates yielded a solution having a H ion concentration of pH 6.8. Sufficient amount of this was added, sterile, to the sterile media to give a concentration of 1 per cent of the dry salt mixture, or approximately M/7. The pH of the final media was 6.8. All of the media were tubed in 8 by 1 inch test tubes, 40 cc. to the tube. Inoculation was made from twenty-four-hour slant agar cultures, except in the case of the two anaerobes where inoculations were made directly from broth cultures. Efforts were made to make the amounts of inoculum small and as nearly uniform as possible.

Incubation was conducted at the optimum temperatures of the given organisms.

The metabolic changes were determined as follows:

Total nitrogen, by the Gunning modification of the Kjeldahl procedure

Non-protein nitrogen, by the Folin and Wu method for the determination of non-protein nitrogen in the blood

Protein nitrogen, by subtracting the non-protein from the total nitrogen figure

Ammonia nitrogen, by Van Slyke and Cullen's modification of the Folin air current method

Amino nitrogen, by the micro-method of Van Slyke (1913–1914, 1915) and by the Brown modification (1923) of the technique of Henrique and Sörensen

Quantitative Biuret, by the method of Vernon (1904)

Glucose, by the method of Benedict (1911), involving the use of potassium thiocyanate and copper sulphate

H Ion Concentration, by the colorimetric method of Clark and Lubs (1917)

Turbidity (approximate), by means of the McFarland nephelometer system (1907) and a comparator block

In a preliminary study of the influence of glucose on the metabolism of *B. subtilis*, *B. cereus*, *Proteus vulgaris* and *Bact. coli*, without added buffer, the following observations were made.

B. subtilis, a glucose-utilizing organism, is able to elaborate a proteolytic enzyme in the presence of glucose and to effect nitrogenous changes which are comparable or may even exceed those produced in plain peptone. The amino nitrogen figures for the 0.5 and 1.0 per cent peptone containing 1 per cent glucose were higher than for the plain peptone tubes, while the ammonia figures were variable, but ran fairly parallel in both media.

On the other hand, *B. cereus*, a closely related member of the subtilis group, presented a different type of nitrogen metabolism. The glucose was rapidly fermented and a H ion concentration inimical to proteolytic activity was soon reached. In plain peptone the nitrogenous changes closely resembled those of *B. subtilis*, that is, *B. cereus* brought about considerable increase in amino and ammonia nitrogen; in the glucose peptone solution, however, very little increase could be demonstrated.

Proteus vulgaris also produced a distinct acidity in glucose peptone which was sufficient markedly to inhibit proteolytic activity. There was an actual apparent loss of amino and ammonia nitrogen in the glucose medium, which may perhaps be explained by the assumption that the simple amino acids and ammonia were used

for structural purposes, with no compensatory production of these substances.

Glucose exerted a restraining action on *Bact. coli* quite similar to that of *Proteus vulgaris*. There was abundant evidence that this organism also utilized ammonia for growth, in the presence of glucose, and in some instances reduced it to zero. On the other hand, *Bact. coli* caused a distinct increase in amino and ammonia nitrogen in the glucose-free medium.

In a second preliminary experiment simple comparative metabolic studies were made on *B. anthracis*, *Bact. coli* and *Bact. paratyphosum* A and B, in plain and in glucose peptone.

The amino, non-protein and ammonia nitrogen figures indicate that the metabolic activities of *B. anthracis* in plain and in glucose peptone are quite similar to those of *B. cereus*. The rapid change in H-ion concentration in the presence of glucose from pH 6.9 to pH 4.8 within twenty-four hours readily explains why metabolic activity of *B. anthracis* is so profoundly influenced (retarded) by the glucose.

The nitrogenous changes produced by *Bact. coli* in plain peptone were slight as compared with those of the subtilis group. There was a noticeable increase in amino, ammonia and non-protein nitrogen between the tenth and the thirtieth days of incubation, whereas a decrease in these substances was observed in the glucose-containing tubes. This again is to be expected from the rapid increase in H-ion concentration to pH 4.6 in twenty-four hours.

The nitrogenous metabolism of *Bact. paratyphosum* A and B was quite similar in many respects to that of *Bact. coli*. Amino nitrogen decreased in both the plain and glucose peptone during the first ten days. After this there appeared to be an increase. The small amount of ammonia present at the beginning in the glucose-containing medium disappeared completely, while in the plain medium some increase took place.

RESULTS OBTAINED IN EXPERIMENTS INVOLVING THE USE OF BUFFER

The phosphate buffer exerted a marked influence on the nitrogen metabolism of many of the organisms studied. Its effect on

closely related types or species varied widely in a number of instances, as, for example, with certain members of the subtilis group.

Bacillus subtilis, Bacillus cereus and Bacillus megatherium

B. subtilis and *B. cereus* produced free ammonia more rapidly in the buffered sugar-free medium than in the plain (figs. 1, 2 and 3), whereas little ammonia was formed by *B. megatherium* in the buffered plain peptone water, but an appreciable amount in the unbuffered (fig. 4).

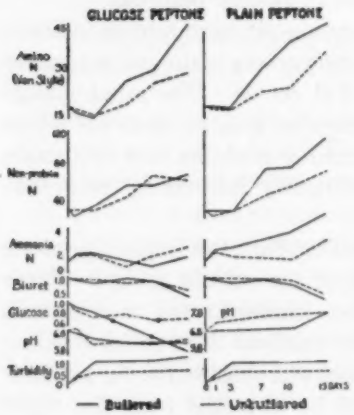

FIG. 1

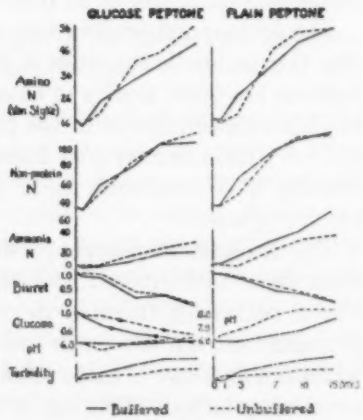

FIG. 2

FIG. 1. BACILLUS SUBTILIS (SHELTEN)
FIG. 2. BACILLUS SUBTILIS (TAP WATER)

Similarly, the amino nitrogen figures for both *B. subtilis* and *B. cereus* were generally higher for the buffered tubes than the unbuffered, while those of *B. megatherium* were appreciably lower.

B. subtilis revealed essentially the same kind and degree of changes in glucose as in plain peptone, and in the buffered glucose as in the plain glucose medium. *B. cereus* and *B. megatherium*, on the other hand, were influenced very little by the phosphate in the glucose medium, and there was comparatively little proteolytic action in both the buffered and the unbuffered glucose

peptone water, due to the rapid increase of H-ion concentration. It may be assumed that the unfavorable influence of the phosphate on the metabolism of *B. megatherium* in the sugar-free medium is due to the slower action of the proteolytic enzyme at the lower H-ion concentration, pH 6.8.

The biuret and non-protein nitrogen figures are in agreement with the changes indicated by the free ammonia and amino nitrogen determinations. Glucose consumption was in all three instances greater in the buffered than in the unbuffered solutions.

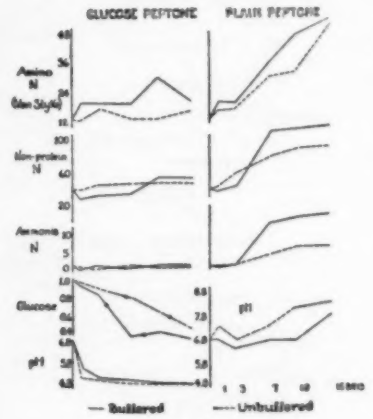

FIG. 3

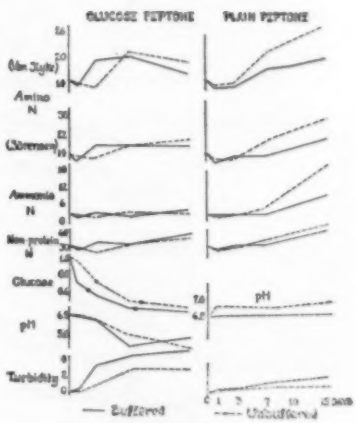

FIG. 4

FIG. 3. BACILLUS CEREUS (HAY INFUSION)
FIG. 4. BACILLUS MEGATHERIUM (N. Y. U.)

The growth curves, when given, correlate with those showing glucose consumption; in other words, the balanced phosphate favored bacterial development.

Erythrobacillus prodigiosus

Erythrobacillus prodigiosus showed a type of nitrogen metabolism in unbuffered plain and glucose peptone very similar to that indicated for *B. cereus* (see fig. 5). In the former medium a rapid increase in amino, non-protein, and ammonia nitrogen occurred, while in the unbuffered glucose peptone tubes virtually

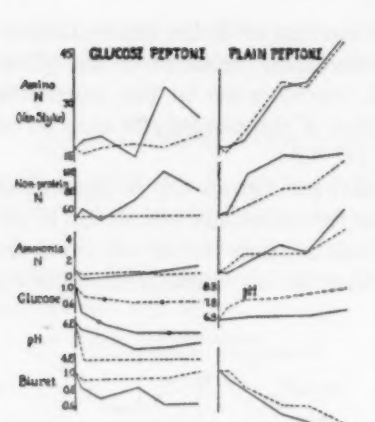

FIG. 5

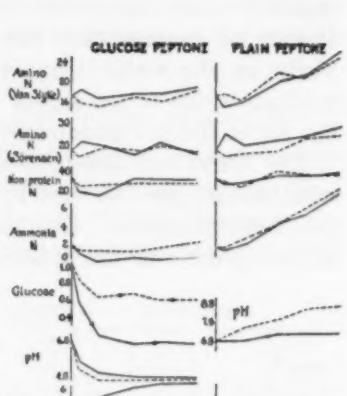

FIG. 6

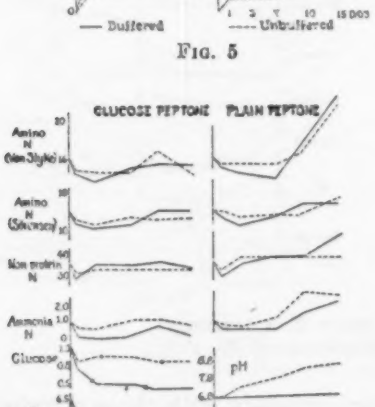

FIG. 7

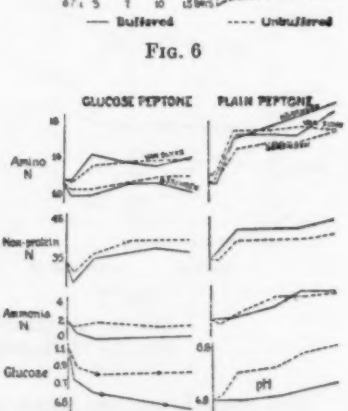

FIG. 8

FIG. 5. ERYTHROBACILLUS PRODIGIOSUS
FIG. 6. BACTERIUM COLI
FIG. 7. BACTERIUM TYPHOSUM (R)
FIG. 8. BACTERIUM PARATYPHOSUM A (Kr. 207)

no changes could be detected in these nitrogen fractions. The rapid drop in pH, from 6.8 to 4.8, within twenty-four hours explains the lack of proteolytic activity in the sugar-containing cultures. Added phosphate, however, prevented this rapid change in H-ion concentration and elicited considerable proteolytic action in the presence of glucose.

A distinct increase in amino and non-protein nitrogen was observed in the buffered glucose-containing cultures, with a simul-

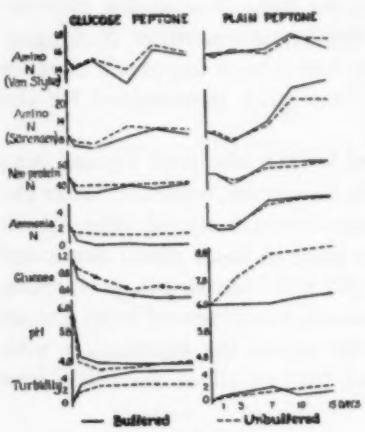

FIG. 9

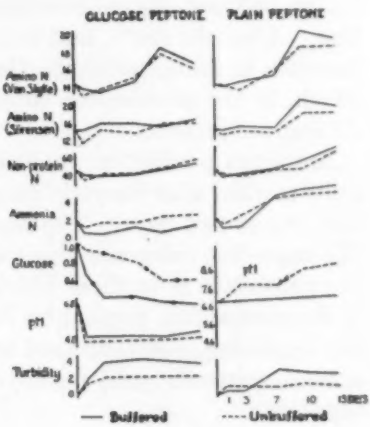

FIG. 10

FIG. 9. BACTERIUM PARATYPHOSUM B (J-12)
FIG. 10. BACTERIUM PULLORUM (Y-24)

taneous decrease in the biuret figures. Glucose utilization was approximately three times as great in the buffered tubes as in the unbuffered, and the turbidity values were noticeably higher.

The addition of phosphate to the plain medium resulted in a slight acceleration of the nitrogen metabolism of this organism. The more luxuriant growth in the buffered tubes seems to be directly associated with the favorable regulatory influence of the phosphate on OH ion concentration.

Bacterium coli, Bacterium typhosum, Bacterium paratyphosum A, Bacterium paratyphosum B, and Bacterium pullorum

These five organisms are grouped together here because of their general relationships and the close similarity, on the whole, of their metabolic activities (see figs. 6, 7, 8, 9 and 10).

All showed a decrease, though in some instances very slight, in the earlier stages, in amino, non-protein, and ammonia nitrogen. *Bact. typhosum* and *Bact. paratyphosum* A and B utilized more nitrogen in the buffered glucose-containing medium than in the glucose-free. After the early, and in a few instances rather prolonged, decrease in amino nitrogen, there was a very apparent increase which, in the glucose-free tubes, was quite pronounced for the different organisms.

Ammonia production, as judged by the observed figures, was quite marked after the early drop in the curves, especially after the first one or two days. The increase in accumulated ammonia in the sugar-free tubes was about the same in those which contained the phosphate as in those which did not. Ammonia production in the glucose-free peptone by *Bact. coli* was apparent from almost the beginning, and continued to the end of the experiment, with a total ammonia yield far beyond that of all of the other four organisms.

Glucose utilization was in every instance much greater in the phosphate-containing than in the unbuffered tubes. The buffer also reacted very favorably on the growth of all of the five organisms in the glucose-containing tubes.

The amount of phosphate used in the sugar-containing peptone was not sufficient to prevent marked increase in H-ion concentration, though a slight buffering action was apparent. On the other hand, the buffer maintained an almost constant pH in the glucose-free tubes throughout each of the experiments.

Proteus vulgaris

Proteus vulgaris displayed metabolic changes in buffered glucose peptone which in the main were quite similar to those in the plain peptone (see fig. 11). Apparently the amount of phos-

phate added proved to be sufficient to maintain the H-ion concentration within suitable growth limits and to favor continued normal nitrogen metabolism. Free ammonia production in the buffered glucose medium approximated that which occurred in the plain peptone, but in the unbuffered glucose broth little change took place. The initial decrease in free ammonia may be ascribed to utilization of that nitrogen fraction by the bacteria for structural purposes.

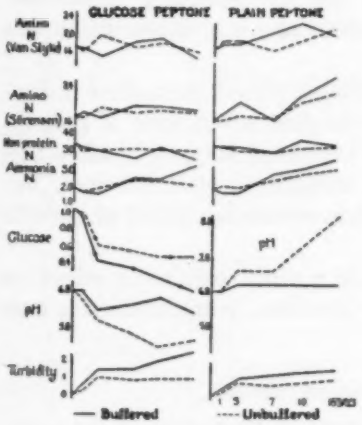

FIG. 11

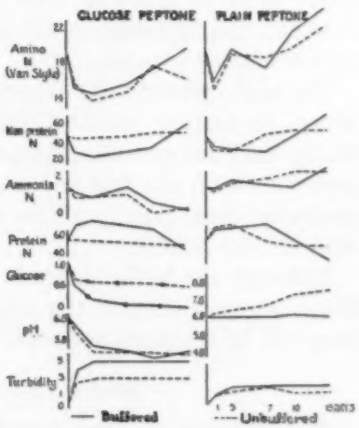

FIG. 12

FIG. 11. PROTEUS VULGARIS (N. Y. U.)
FIG. 12. STAPHYLOCOCCUS AUREUS

Somewhat higher amino, non-protein and ammonia nitrogen values were found in the buffered plain broth cultures than in the unbuffered. Both growth and glucose utilization were greater in the cultures which contained added phosphate than in those which did not. The acceleration of metabolic activities apparently bears a direct relation to the favorable pH maintained by the balanced phosphate mixture.

Staphylococcus aureus

The metabolism of *Staphylococcus aureus* was similar in many ways to that of the colon-typhoid group (fig. 12). During the

first seven days of growth utilization of amino nitrogen in all of the tubes was very pronounced, amounting to approximately 20 per cent of the total amino nitrogen content of the control medium. The buffer exerted little influence in either the plain or the glucose medium. Free ammonia decreased noticeably and practically disappeared from both glucose cultures by the fifteenth day.

The turbidity of the buffered glucose-containing culture was about twice as great as that of the corresponding unbuffered tube and the difference in the amount of sugar consumption was approximately the same.

The plain peptone cultures of *Staph. aureus* gave some indication of proteolytic activity after the first seven days of growth. Amino and non-protein nitrogen increased noticeably after this period in both the buffered and unbuffered plain medium, but somewhat more rapidly in the tubes containing added phosphate than in the others.

Although the buffer exerted only a slight regulatory effect on H-ion concentration in the sugar medium, it maintained a uniform pH in the plain peptone.

Bacterium bronchisepticum

Although the nitrogen metabolism of *Bacterium bronchisepticum* was slight, it proved to be unique in respect to changes in the amino nitrogen fraction (fig. 13). The Van Slyke figures show a progressive decrease in amino nitrogen with a slight concomitant increase in free ammonia. The lower non-protein nitrogen values of the buffered cultures are to be expected, in view of the much more luxuriant growth obtained in the buffered medium, both the glucose and the plain. The increased growth seems to be directly associated with the maintenance of a favorable pH by the buffer during the entire fifteen-day period of observation, the phosphate holding the H-ion concentration at or near pH 6.8 as compared with an increase in pH to 8.0 or slightly above in the unbuffered medium.

The presence of glucose did not affect the metabolism of this organism. Glucose determinations were made here, but it is

quite probable that there was very little glucose utilization at best. The increased turbidity in the buffered peptone tubes may be ascribed, in a small measure, to the phosphate as such, aside from its buffer action.

Pseudomonas pyocyanea

Ps. pyocyanea exhibited a unique type of nitrogen metabolism in the presence of added phosphate (fig. 14). The changes brought about by this organism in amino and non-protein nitrogen in the

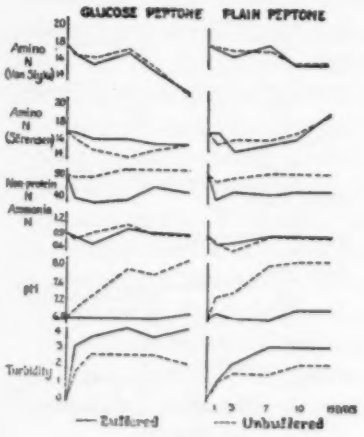

FIG. 13

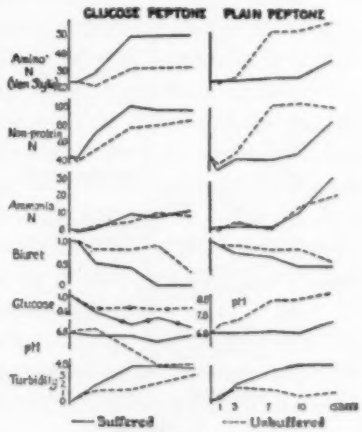

FIG. 14

FIG. 13. BACTERIUM BRONCHISEPTICUM
FIG. 14. PSEUDOMONAS PYOCYANEA (N. Y. U.)

buffered glucose peptone were quite pronounced, and approximated those occurring in the unbuffered sugar-free medium. On the other hand, in the unbuffered glucose peptone the proteolytic action of this organism was relatively small. Biuret disappeared much more rapidly in the buffered than in the unbuffered tubes, and glucose utilization was also much greater in the former.

The presence of added phosphate in the plain peptone apparently retarded the proteolytic activity of *Ps. pyocyanea*, if amino and non-protein nitrogen are taken as an index. Production of free ammonia was approximately the same in the buffered and

unbuffered glucose-free peptone. In the peptone containing no sugar the ammonia remained at or near zero until after the seventh day when there was a marked and continuous rise. The total increase, however, was greater in the buffered medium.

It is interesting to note that the added phosphate exerted a strong regulatory action on pH in both the plain and glucose peptone cultures, and encouraged greater luxuriance of growth, as indicated by turbidity.

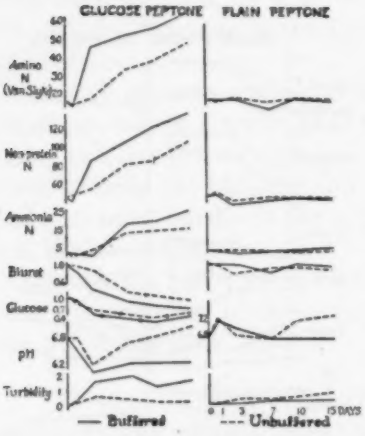

FIG. 15

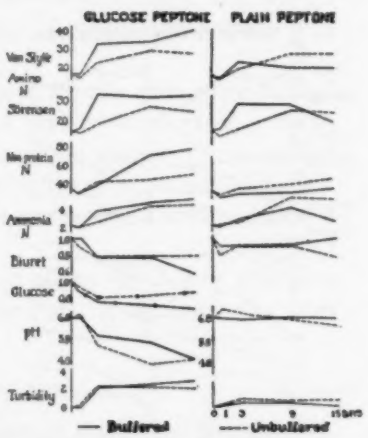

FIG. 16

FIG. 15. CLOSTRIDIUM SPOROGENES
FIG. 16. CLOSTRIDIUM WELCHII

Clostridium sporogenes

Added phosphate markedly accelerated the metabolism of this anaerobe in glucose peptone (fig. 15). The amino and non-protein nitrogen increase was not only much more rapid in the buffered glucose medium during the first three days of growth, but continued to be approximately twice that of the corresponding unbuffered glucose-containing tubes during the entire fifteen-day period of observation.

In agreement with the changes in amino and non-protein nitrogen, the biuret fraction disappeared more rapidly from the

buffered tubes than from the other. More abundant production of free ammonia also occurred in the buffered carbohydrate medium. Turbidity figures indicate that growth was much more luxuriant in the buffered glucose peptone tubes than in the unbuffered.

In the plain medium *Cl. sporogenes* gave a scanty growth, and produced no appreciable changes in the various nitrogen fractions.

Clostridium welchii

Although the nitrogen changes produced by *Cl. welchii* in buffered glucose peptone were not so extensive as those brought about by *Cl. sporogenes*, the response of this anaerobe to added phosphate was similar in nature (fig. 16). Figures for amino nitrogen and non-protein nitrogen, as well as free ammonia, were higher in the buffered glucose peptone than in the unbuffered sugar medium, and corresponding differences in biuret reduction were apparent. With this increased peptolytic activity, it is interesting to note the favorable influence of the phosphate buffer in preventing the rapid increase in H-ion concentration which took place in the unbuffered glucose peptone.

Growth in the carbohydrate-free medium was slight, showing the dependence of this organism on sugar for energy requirements. No material differences were observed in the two sugar-free media.

GENERAL DISCUSSION

Although phosphate buffer accelerated the growth and metabolic activities of many of the organisms, it did not affect all members of the same group, or even closely related species in the same manner. For example, members of the subtilis group varied in their response to added phosphate in both plain and glucose-containing peptone solutions. *B. subtilis* and *B. cereus* produced more free ammonia in the buffered sugar-free medium than in the unbuffered plain peptone, whereas *B. megatherium* formed little free ammonia in the buffered plain medium, but large amounts in the unbuffered.

B. subtilis displayed marked increases in amino and non-protein nitrogen, whether grown in plain or in glucose peptone. *B.*

cereus and *B. megatherium*, on the other hand, gave little evidence of proteolytic changes in the glucose peptone on account of the rapid increase in H-ion concentration. The greater activity *B. subtilis* in glucose peptone is without doubt due to its ability to utilize non-carbohydrate carbon for energy about as readily as that of glucose, and thus to maintain a reasonable acid-alkali balance.

E. prodigiosus resembled *B. cereus* in the nitrogenous changes produced in the unbuffered media, but it differed from *B. cereus* in the buffered glucose medium. Its peptolytic activity, as shown by the amino nitrogen, non-protein nitrogen, and biuret changes, was considerable in the buffered glucose-containing cultures, but very slight in the unbuffered sugar medium. The rapid drop from pH 6.8 to 4.8 which occurred in the unbuffered tubes of both *B. cereus* and *E. prodigiosus* explains the lack of change in the various nitrogen fractions of these cultures, while the maintenance of a more favorable pH in the buffered medium permitted considerable peptolytic action. Glucose utilization was greater in the buffered tubes of *E. prodigiosus* than in the unbuffered, as was also the case with *B. cereus* and *B. megatherium*.

In contrast to the marked peptolytic activity of the subtilis group, members of the coli-typhi-paratyphi group exhibited a relatively slight nitrogen metabolism. *Bact. pullorum* and *Bact. typhosum* resembled each other closely in their metabolic activities. A decrease in amino nitrogen was generally observed in the glucose peptone cultures, while in the sugar-free media a noticeable increase in that fraction occurred after the first three days' of growth. The buffered plain peptone cultures did not differ very greatly from the unbuffered, although there was some indication of greater proteolytic activity in the former.

Bact. coli, like *Bact. typhosum*, showed little nitrogenous change in the glucose-containing medium, but in plain peptone this organism was decidedly more active than *Bact. typhosum*. The nitrogen metabolism of *Bact. paratyphosum* A and B was in many ways similar to, but more pronounced, than that of *Bact. typhosum* and *Bact. pullorum*.

It is interesting to note that in the buffered glucose-containing

cultures of *Bact. coli*, *Bact. pullorum*, *Bact. typhosum*, and *Bact. paratyphosum* A and B, more sugar was utilized than in the unbuffered, and along with this change a more luxuriant growth and a greater decrease in free ammonia was observed. The amount of phosphate buffer added to plain peptone cultures of this group exerted a strong regulatory effect on H-ion concentration, but in the glucose medium the buffer checked the rapid drop in pH to only a small degree.

The addition of one per cent phosphate to cultures of *Proteus vulgaris* proved sufficient to maintain a favorable H-ion concentration, even in the presence of glucose, and to encourage continued nitrogen metabolism. Amino nitrogen values were as a rule higher in the buffered cultures. Free ammonia production was greater in the buffered media, irrespective of the presence or absence of glucose, and in the unbuffered glucose-peptone cultures only a slight increase took place.

Staph. aureus in glucose peptone showed a greater decrease in the amino nitrogen fraction than did the coli-typhi group. In plain peptone an initial decrease in amino nitrogen occurred, followed by an increase. Ammonia nitrogen also decreased somewhat during the entire fifteen days of growth in the glucose-containing cultures. The added phosphate checked the rapid drop in pH to a small extent.

Bact. bronchisepticum with its slight metabolic activity more or less resembles the colon group of organisms, but proved to be unique in that it showed a progressive decrease in amino nitrogen during the fifteen day observation period.

Cl. sporogenes showed a much greater increase in amino and non-protein nitrogen and in free ammonia in the buffered glucose peptone than in the unbuffered, with more luxuriant growth in the former tubes. The metabolic end products of this anaerobe were such that the total pH reduction in the glucose-containing media was small.

Cl. welchii also displayed more peptolytic activity in buffered glucose peptone than in the unbuffered. This seems to be directly associated with the regulatory effect of the added phosphate on H-ion concentration, for turbidity estimations indicated similar

amounts of growth. Amino and non-protein nitrogen, and free ammonia production proved to be decidedly greater in the buffered than in the unbuffered glucose medium.

CONCLUSIONS

Phosphate buffer when present in reasonable amount (1 per cent) will in many instances maintain a sufficiently low level of H-ion concentration to permit the given organisms to bring about nitrogenous changes in the substrate as though no utilizable carbohydrate were present.

The ability to carry on such activities varies widely with different bacterial groups or species, and even with members of the same species.

The methods employed in this investigation make it possible to study nitrogen metabolism over a wide range, as indicated by the comparative determinations of total, protein, non-protein, ammonia, amino and polypeptid nitrogen, and biuret.

Aside from the influence of buffer on nitrogen metabolism, it was hoped to make this study of considerable interest in furnishing definite information concerning the activities of 22 different, well-known species, not only as to nitrogen changes, but as to the action of these organisms on glucose, and the resultant changes in H-ion concentration under the different experimental conditions.

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THE INFLUENCE OF SODIUM CHLORIDE ON THE GROWTH AND METABOLISM OF YEAST

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The effects of the commoner salts on the physiology of the higher animals and plants have been studied by numerous investigators. In order to obtain quantitative data workers in the animal field, e.g., Loeb (1906), Lillie (1901), and Moore (1901), have studied normal rhythmical movements such as heart beat, the contractions of Polyorchis or the contractions of the lymph hearts of frogs. This type of research provided the experimental basis for several important conclusions: (*a*) in low concentrations the common salts exert a favorable and stimulating effect, (*b*) a toxic effect is exerted in higher concentrations, and (*c*) the influence of a salt is modified by the presence of other salts in the solution. In the case of higher plants investigators have concerned themselves particularly with the influence of salts on the dry weight of the plant.

The unicellular organisms provide a means of analyzing some of these effects in greater detail by chemical methods. Already there is a considerable literature dealing with this branch of the field. Several workers have shown recently that low concentrations of many different salts stimulate the growth of bacteria. Hotchkiss (1922) in a study of *B. coli* reported that 15 of a total of 23 chlorides stimulated growth as measured by the turbidity method. This was true of those salts which are usually considered to be particularly toxic. Holm and Sherman (1921) found that 0.2 M concentration of NaCl hastens the appearance of turbidity in media inoculated with *B. coli*. No measurements were made of total growth. Brooks (1919) made the important observation that an increased output of CO_2 by *B. subtilis* took place in the

presence of 0.15 M NaCl, 0.2 M KCl or 0.05 M $CaCl_2$. She also observed the antagonistic effects when mixtures were used. Gustafson (1919) made similar observations in connection with *Aspergillus niger*. Lipman (1909) in an earlier research had found that nitrification and ammonification by bacteria were stimulated in a similar manner. Mitra (1917) investigated the effect of chlorides, singly and in mixtures, on the vegetative reproduction of a wine yeast growing in a synthetic medium. Larger crops of yeast were obtained when low concentrations of different salts were added.

We have attempted a more detailed investigation of the influence of several chlorides on the following characteristics of a yeast fermentation: (*a*) vegetative growth, (*b*) nitrogen content of crop, (*c*) rate of CO_2 production and total output, and (*d*) the quantitative relationship between several of these characteristics.

METHODS

The organism used was a type of *S. cerevisiae* used in a local brewery. Pure cultures were maintained by the usual bacteriological methods.

Medium

In choosing a medium for work of this nature it is impossible to avoid the complications due to "antagonism" between the normal salt content of the medium and the salt under investigation. It is obvious that in all the microbiological work which we have reviewed this factor is present to an undetermined extent, and this is particularly so when synthetic media were used. Mitra's (1917) medium contained 0.1 per cent ammonium phosphate, and his experimental findings include the effect of 0.058 per cent NaCl. Recognizing this difficulty we have used wort made according to a carefully standardized method. This medium contained approximately 0.25 per cent of salts, but it gives a control crop of yeast which is more than 10 times the maximum crop obtained by Mitra as a result of stimulation. In other words any changes observed in our experiments are changes from a normal and not from an abnormal type of fermentation. Furthermore the

effects which we regard as significant are produced by large amounts of added salt, and not by quantities significantly less than the initial salt content of our medium.

Our medium was prepared as follows: Extract 600 grams of fresh malt with 3000 cc. of distilled H_2O at 60°C. for three hours. Strain through cheese-cloth, and sterilize for one and one-half hours at 15 pounds steam pressure. Remove the precipitate by filtration, and sterilize for twenty minutes. Determine the sugar concentration by the Shaffer-Hartmann method. Our standard medium was made up to contain 10 per cent sugar. The various amounts of NaCl were added to the wort in the experimental tubes previous to the final sterilization, except in certain experiments when the salt was held in a special container above the medium in the tubes.

Cultures

The experimental cultures were contained in bent tubes similar to that described by Fraser (1921). The bath surrounding the moving rack was maintained at 26.5°C. The upright arm of the culture tube passed through an arc of 20° 50 times per minute. Each tube contained 70 cc. of medium, and the volume of the horizontal arm was approximately 80 cc. (see fig. 1). The CO_2 given off was absorbed without loss in large test-tubes containing NaOH. Specially designed absorption tubes were used. The amount of CO_2 taken up by the soda was determined by a double titration of an aliquot using a standard HCl, phenolphthalein and methyl orange. The efficiency of these methods will be more clearly demonstrated by the results recorded for several controls.

The objection might be raised that the CO_2 absorbed by the NaOH is not quantitatively representative of the amount produced at any given time owing to, (*a*) the solubility of CO_2 in the medium, and (*b*) the different solubilities of CO_2 in media containing various concentrations of NaCl. We consider, however, that the findings of Findlay and co-workers (1910; 1912; 1913) on the solubility of CO_2 in H_2O and solutions of KCl support our conclusion that CO_2 is not held by the medium under the

experimental conditions outlined above. We desire to emphasize the necessity for adequate and uniform motion in the culture tubes throughout the entire period of the experiment. Our experience has been that only results of limited value are obtained when the shaking is performed by hand or by a variable-speed motor.

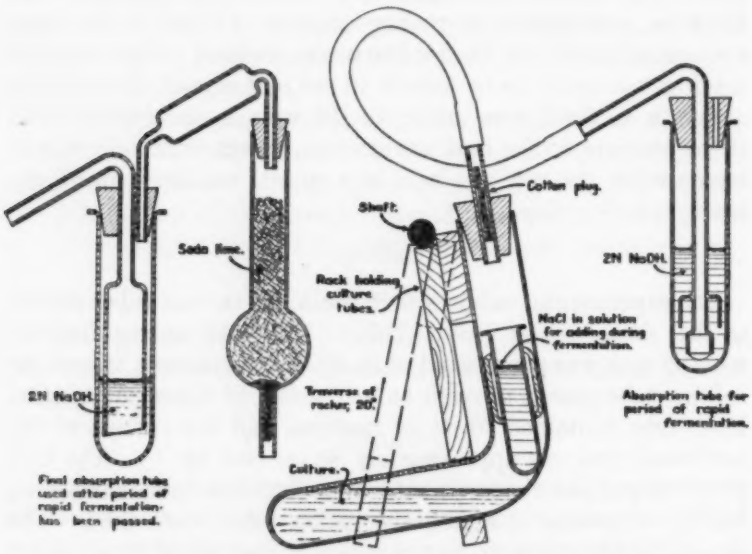
Cotton plug.
Shaft.
Soda lime.
2N NaOH.
Rack holding culture tubes.
NaCl in solution for adding during fermentation.
Traverse of rocker, 20".
2N NaOH.
Absorption tube for period of rapid fermentation.
Culture.
Final absorption tube used after period of rapid fermentation has been passed.

FIG. 1. ROCKER TUBE FOR CULTURE. ABSORPTION TUBES FOR CARBON DIOXIDE

Inoculum

The culture used to inoculate the experimental tubes was grown in 2 per cent wort for twenty-four hours on the moving rack. Each tube received 1 cc., 2 cc. or 5 cc. of culture accurately measured. In order to obtain agreement among controls inequalities in the number of cells added must be avoided, and this cannot be achieved if the yeast in the parent culture is flocculent.

Yeast crops

Alundum crucibles (Norton, porosity R. A. 360) were used to remove the crops from the fermented wort. Controls showed an agreement to within 2.0 per cent by weight after two hours in an oven at 100°C. To maintain this agreement it is necessary to standardize the washing of the crops. Hot cleaning solution will prepare the crucibles for further experiments, but their porosity becomes too great after 4 or 5 experiments.

TABLE 1

SERIES	SUGAR IN WORT	FERMENTATION PERIOD	NaCl	YEAST
	per cent	*hours*	*per cent*	*grams*
1	10	61	0	0.4423
			0	0.4440
			0.02	0.4224
			0.05	0.4330
			0.10	0.4177
			0.50	0.4025
2	7	112	0	0.3839
			0	0.3942
			0.5	0.3674
			0.5	0.3467
			1.5	0.2970
			1.5	0.2887
3	8	112	0	0.4204
			0	0.4179
			2.0	0.3156
			4.0	0.1611
			7.0	0.0863
			10.0	0.0819

THE INFLUENCE OF SODIUM CHLORIDE ON VEGETATIVE GROWTH

Culture tubes containing 70 cc. of sterile wort free from suspended matter and containing various quantities of NaCl were inoculated with 2 cc. of a twenty-four-hour culture. The tubes were then placed on the rocker, and were allowed to ferment to completion. The crops of yeast obtained were weighed in the dry state, and the results from a few experiments are summarized

in table 1. This range of salt concentration has been covered several times, and the above results are characteristic. They show very definitely that in wort, a medium which is particularly suitable for growth and fermentation, there is a gradual diminution in the size of the yeast crop as the concentration of NaCl is increased. We have no convincing evidence of a concentration which acts as a stimulant.

TABLE 2

CONTROL	AGE OF CULTURE		CO_2 PER HOUR	CO_2 IN FORTY-EIGHT HOURS
	hours	*minutes*	*grams*	*grams*
1	13		0.1758	2.068
	14		0.2070	
	15	25	0.2070	
	16		0.2034	
	17		0.1842	
2	13	37	0.1704	2.070
	14	36	0.2028	
	15		0.2100	
	16		0.1968	
	17		0.1650	
3	12	48	0.1278	2.010
	13	49	0.1806	
	14	48	0.2070	
	15	48	0.2040	
	16	49	0.1986	

THE INFLUENCE OF SODIUM CHLORIDE ON GAS PRODUCTION

Experiment I

Before proceeding to investigate the influence of NaCl on the rate of CO_2 evolution and on the total CO_2 production it was necessary to satisfy ourselves that a satisfactory degree of uniformity could be obtained in control fermentations and in our methods of measurement. Accordingly at regular intervals during the progress of this research two or more controls have been followed under standard conditions. Unless the same degree of accuracy was obtained in the controls the results were discarded.

Tubes containing 70 cc. of 10 per cent wort were inoculated with 5 cc. of a twenty-four-hour culture in 2 per cent wort. The CO_2

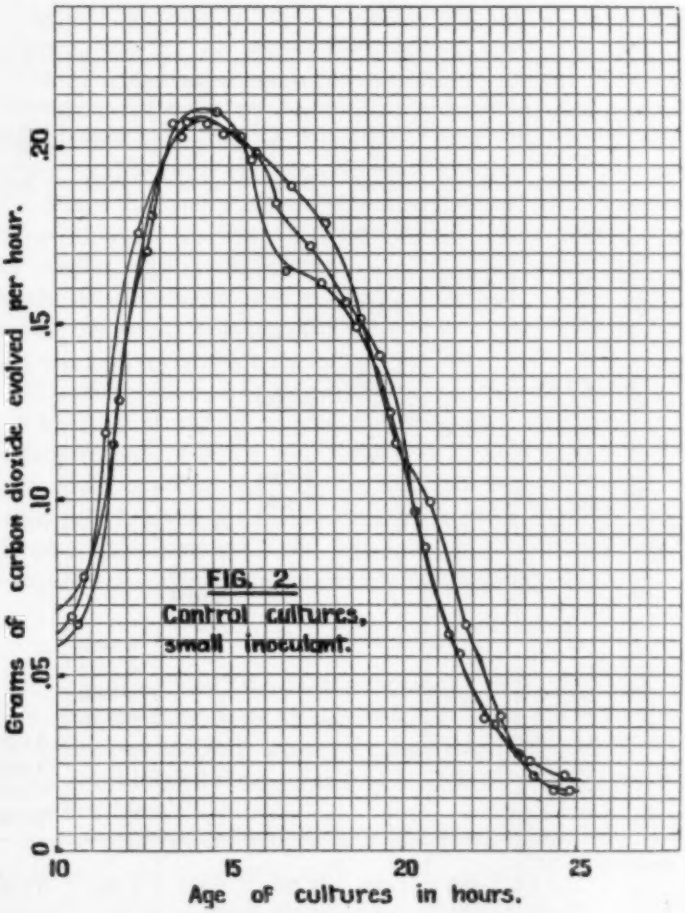

FIG. 2. CONTROL CULTURES, SMALL INOCULANT

given off was determined every hour for each tube, and the total figures for a forty-eight-hour period were also obtained.

The curves in figure 2 show the extent of the agreement between the results as a whole, and in table 2 the rates at the peaks of the curves are given. Total yields of CO_2 are included in the table.

TABLE 3

AGE OF CULTURE		CO_2 PRODUCTION PER HOUR					
		Control	1 per cent NaCl	2 per cent NaCl	3 per cent NaCl	4 per cent NaCl	5 per cent NaCl
hours	*minutes*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*
13		0.0706					
13	40		0.1090				
13	50			0.0727			
14	10	**0·1300**					
14	20		**0·1880**				
14	45			0.1210			
15	30	0.1210					
15	40		0.1770				
15	50			0.1350			
16	39	0.0835					
16	45		0.1270				
16	53			**0·1380**			
17	50			0.1260			
20					0.0712		
21					0.0788		
22					0.0790		
23					**0·0815**		
24					0.0762		
32	10					0.0496	
33	10					0.0521	
34	10					**0·0560**	
35	10					0.0445	
41	20						0.0499
42	20						**0·0554**
43	20						0.0531
44	20						0.0461
CO_2 produced in 107 hours.....		1.473	1.560	1.701	1.362	1.238	0.896

Experiment II

Six tubes containing 70 cc. of wort, 10 per cent sugar, were prepared and sterilized. The first tube was a control, and the

remainder contained from 1 to 5 per cent NaCl. Each tube was inoculated with 1.0 cc. of a twenty-four-hour culture. The rates of CO_2 evolution and the total yields were determined. In table 3 we have grouped together the rate figures for each fermentation in the region of maximum gas evolution. The following conclusions may be made from these observations. This strain of yeast will grow and ferment in wort containing 10 per cent sugar and up to 5 per cent NaCl. The maximum rate of gas production is raised by the presence of 1 to 2 per cent of NaCl, and the total production is also greater than in the control. Above this salt concentration the maximum rate becomes steadily smaller, and in the concentrations used the time required to reach the maximum rate gradually increases. This flattening out of the rate curves is partially due to a steady increase in the initial lag phase of the fermentation period.

The stimulation of CO_2 production by low salt concentrations has been confirmed by numerous experiments of this type. The concentrations of NaCl used have been varied up to 2 per cent. Individual experiments vary in the concentration within this range which gives the highest maximum rate, but they agree in showing that between 1 and 2 per cent of NaCl bring about an increase in the maximum rate and total CO_2 production.

Experiment III

In this experiment the tubes were inoculated with 5 cc. of culture, and the same type of quantitative observations were made. Six tubes were studied: 2 controls, 2 containing 0.5 per cent NaCl, and 2 containing 1.0 per cent NaCl. The important figures are given in table 4.

In the presence of 0.5 per cent NaCl the lag-phase is slightly prolonged, the maximum rate of CO_2 evolution is raised, and total CO_2 production is greater than in the controls. When the concentration of salt is raised to 1.0 per cent we find no satisfactory evidence of a stimulation either of the rate or total CO_2 figures. The lag-phase is prolonged, but otherwise the results fall within the range of variation which is possible with controls.

The results for the 1.0 per cent fermentations are therefore essentially different from those obtained in experiment II, and attention is called to the only change in the conditions, namely, an increase from 2 to 5 cc. in the inoculum used.

TABLE 4

CULTURE	AGE		CO_2 PER HOUR	CULTURE	AGE		CO_2 PER HOUR
	hours	*minutes*	*grams*		*hours*	*minutes*	*grams*
Control	18	30	0.213	Control	18	40	0.234
	19	30	0.294		19	40	0.292
	20	30	0.298		20	40	0.294
	21	30	0.297		21	40	0.259
	22	30	0.268		22	40	0.191
	23	30	0.181		23	40	0.092
0.5 per cent NaCl	18	50	0.233	0.5 per cent NaCl	19		0.207
	19	50	0.293		20		0.260
	20	50	0.314		21		0.276
	21	50	0.313		22		0.297
	22	50	0.310		23		0.310
	23	50	0.253		24		0.285
1.0 per cent NaCl	19	10	0.220	1.0 per cent NaCl	19	20	0.285
	20	10	0.269		20	20	0.288
	21	10	0.288		21	20	0.258
	22	10	0.296		22	20	0.167
	23	10	0.271		23	20	0.067
	24	10	0.180				

Total CO_2 in 94 hours

Controls, 2.863 grams, 2.756 grams
0.5 per cent NaCl, 2.913 grams, 2.870 grams
1.0 per cent NaCl, 2.824 grams, 2.637 grams

Experiment IV

A still larger number of cells were used to inoculate the experimental cultures. The entire crops from normal control tubes were separated from the media by careful filtration through double filter-cones. The crops were washed with a little distilled water, and dried at the pump. The paper cone and one crop were added to each tube. In previous experiments we had found that

proper agreement was possible among several controls inoculated in this way. The lag-phase is considerably reduced, the peak of gas evolution is reached in about four hours, and the fermentation is completed in twelve hours. Brown (1892; 1894; 1903) used

TABLE 5

CULTURE	AGE		CO_2 PER HOUR	CULTURE	AGE		CO_2 PER HOUR
	hours	minutes	grams		hours	minutes	grams
Control IV A	2		0.256	Control IV A	2	10	0.300
	3		0.440		3	10	0.441
	4		0.561		4	10	0.550
	4	20	0.552		4	30	0.528
	4	40	0.531		4	50	0.531
	5		0.501		5	10	0.507
	5	30	0.498		5	40	0.468
Control IV B	2		0.200	0.5 per cent	2	10	0.196
	3		0.445	NaCl, IV B	3	10	0.467
	3	30	0.590		3	40	0.584
	4		0.608		4	10	0.630
	4	30	0.616		4	40	0.610
	5		0.600		5	10	0.610
	5	30	0.540		5	40	0.540
Control IV C	2		0.224	0.75 per cent	2	10	0.232
	3		0.531	NaCl, IV C	3	10	0.518
	3	30	0.602		3	40	0.618
	4		0.608		4	10	0.618
	4	30	0.664		4	40	0.620
	5		0.608		5	10	0.640
	5	30	0.506		5	40	0.542

Total CO_2

IV A. 48½ hours, Control—2.712 grams, Control —2.707 grams
IV B. 25 hours, Control—2.695 grams, 0.5 per cent NaCl—2.709 grams
IV C. 23½ hours, Control—2.932 grams, 0.75 per cent NaCl—3.019 grams

this method in his studies of normal yeast fermentation, and states that the crop from a given volume of a suitable medium does not increase when it is added to a similar volume of fresh material. We hoped by this technique to be able to study the influence of NaCl on fermentation in the absence of vegetative

reproduction, but we found that under these conditions the crop doubles in weight during the second fermentation. The divergence from Brown's findings is probably due to the fact that our cultures were agitated continuously.

Total crops of yeast were added to tubes of the same batch of medium containing, (*a*) no NaCl, (*b*) 0.5 per cent NaCl, and (*c*) 0.75 per cent NaCl. The rate of gas evolution and the total produced were determined in each case. Experimental results from such a group of cultures are given in table 5. Experiment IV shows quite clearly that when a large amount of yeast is used to inoculate wort containing from 0.5 to 0.75 per cent of NaCl the maximum rate of CO_2 production and the total amount produced are similar to the results obtained in the absence of salt. Similar experiments have been performed in which the salt concentration was raised to 1.0 per cent. In this case both the maximum rate and the total CO_2 figures are depressed. These results as a whole are in striking contrast with our earlier ones obtained from experiments in which a small inoculum was used. We shall discuss the theoretical aspect of these differences late in our report.

Experiment V

An attempt was then made to measure the effects of additions of NaCl which were made during the course of normal fermentations. The salt was carefully weighed, and dissolved in a known volume of distilled water. This solution was placed in a small test-tube which rested in the vertical arm of the fermentation tube (fig. 1). By tilting the apparatus slightly the NaCl could be added at the desired point in the fermentation without additional changes in the conditions of the experiment. By the same method a corresponding volume of H_2O was added to the control fermentation.

The crops of yeast from two normal fermentations were separated, and added to fresh portions of the same medium. The rate of CO_2 evolution was followed until the peaks of the curves were approaching. An addition of 0.5 per cent of NaCl was made to one fermentation. We continued the observations of

rates, and measured the total volumes of CO_2 produced. These figures are given in table 6. These results show that when 0.5 per cent NaCl is added to a normal, vigorous fermentation there is no immediate effect on the rate of CO_2 production. The total amount produced is also unchanged. In a similar experiment the salt was added after the maximum rate had passed, and we observed no stimulation.

Experiment VI

The last experiment was repeated with the exception that 1.25 per cent NaCl was added to one of two cultures before the

TABLE 6

CULTURE	AGE		CO_2 PER HOUR	CULTURE	AGE		CO_2 PER HOUR
	hours	*minutes*	*grams*		*hours*	*minutes*	*grams*
Control	1		0.0460	Control	1		0.0406
	2		0.2369		2		0.2307
	3		0.3415		3		0.3251
H_2O added	3	05		0.5 per cent	3	05	
	3	30	0.3874	NaCl added	3	30	0.3886
	4		0.4204		4		0.4204
	4	30	0.4162		4	30	0.4120
	5		0.3874		5		0.3812
	5	30	0.3700		5	30	0.3648
	6	30	0.2830		6	30	0.3077

Total CO_2 produced

Control, 96½ hours, 2.4476 grams
0.5 per cent NaCl, 96½ hours, 2.4409 grams

maximum rate of CO_2 evolution had been reached. The results from the experiment are represented by curves in figure 3. There is an immediate effect on the rate curve when this amount of NaCl is added to the culture. Previously the two cultures had been almost identical, but the salted fermentation at once developed a rate curve distinctly below that of the control. Later on the control curve cut through the "salted" one, and the fermentation was completed in a shorter period. The total yields of CO_2 were: control 2.3693 grams and 1.25 per cent NaCl 2.3687 grams.

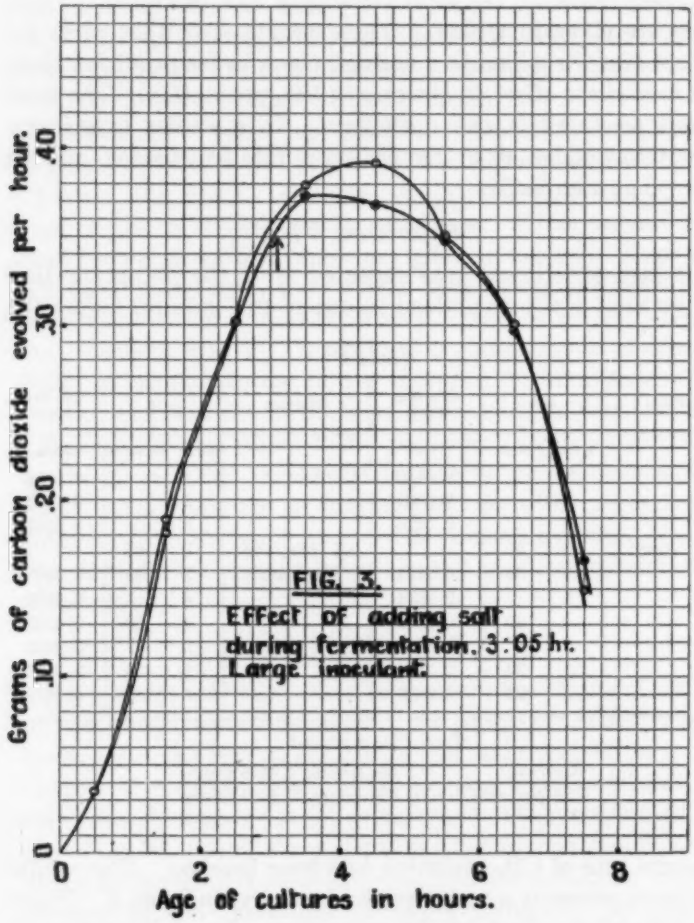

FIG. 3. EFFECT OF ADDING SALT DURING FERMENTATION. 3.05 HOURS. LARGE INOCULANT

THE NITROGEN CONTENT OF YEAST

In order to obtain information regarding the effect of sodium chloride on the chemical composition of the yeast cells the

percentage of nitrogen was determined in crops obtained over a wide range of salt concentrations.

Experiment VII

Six tubes containing 70 cc. of medium were inoculated with 2 cc. of a culture in 10 per cent wort. The crops at the end of thirty-eight hours were removed from the control cultures and those containing 2 per cent of NaCl. After washing and centrifuging 3 times the material was placed in separate, clean tubes, and dried in the oven for six hours at 100°C. Portions of the brittle mass were removed from each tube, and weighed. The crops from cultures containing 4 and 6 per cent of salt were similarly treated

TABLE 7

CULTURE	SAMPLE OF YEAST	NITROGEN IN SAMPLE	NITROGEN IN CROP
	grams	*grams*	*per cent*
Control	0.2474	0.0185	7.47
	0.2256	0.0174	7.72
2.0 per cent NaCl	0.1892	0.0148	7.80
2.0 per cent NaCl	0.1111	0.0087	7.88
4.0 per cent NaCl	0.1947	0.0161	8.27
6.0 per cent NaCl	0.0750	0.0066	8.77

at the end of eighty-five hours. In all cases the N content was determined by the Kjeldahl method. Our methods of procedure had been previously tested by determining the N content of different portions of the same control crop of yeast. Experimental results are given in table 7. Our results indicate a gradual rise in the N content of the crops within this range of salt concentrations. Similar experiments were performed in which the concentration was carried to higher figures, and in these also the N content of the crops was determined. They all agree in showing that when more than 6 per cent of NaCl is present the N figure falls, and when 10 per cent is reached the N figure is similar to that for the controls. We have not the same confidence, however, in these results because under these conditions it is impossible to obtain samples from the dry crops which

are comparable in weight with those from control cultures and those containing only low concentrations of salt. As we have shown earlier in our report the amount of vegetative reproduction is extremely small.

Experiment VIII

The crops from control tubes were isolated, and these were used to inoculate tubes of the same medium containing different concentrations of NaCl. We have found that the control crop in the second fermentation increases in weight approximately 100 per cent. Even in high salt concentrations there is no difficulty in isolating a satisfactory sample of the crop for N determinations. These results are given in table 8. Again we find a gradual rise

TABLE 8

CULTURE	SAMPLE OF YEAST	NITROGEN IN YEAST	NITROGEN IN CROP
	grams	*grams*	*per cent*
Control	0.3839	0.0281	7.34
	0.3646	0.0259	7.10
2.0 per cent NaCl	0.3295	0.0241	7.31
4.0 per cent NaCl	0.1836	0.0136	7.40
6.0 per cent NaCl	0.1135	0.0090	7.94
8.0 per cent NaCl	0.0890	0.0068	7.64
10.0 per cent NaCl	0.1250	0.0086	7.44

and fall in the N content of the yeast crops with the peak of the curve at 6.0 per cent NaCl. In the presence of 10 per cent NaCl little or no growth takes place, and the N content of the yeast crop is similar to that of the control crops. Also the extent of the rise in N is less in this type of experiment than in experiment VII, in which a small inoculum was used. This difference will be considered more fully in our discussion.

THE INFLUENCE OF NaCl ON FERMENTATION RATIOS

It seemed probable that NaCl was influencing the qualitative aspects of the fermentation of wort in addition to its effects on the extent of sugar utilization and gas production. As a prelim-

inary to a more detailed chemical study of the products we have been able to detect a definite change from the normal by a simple calculation of the ratio between sugar utilization and CO_2 output. In addition it is of some importance to measure, if possible, the relative degrees of efficiency of yeast fermenting in different salt concentrations by comparing the weight of sugar utilized with the mass of cells operating. This ratio was used in Pasteur's early investigations to indicate a greater efficiency when yeast fermented under anaerobic conditions. It has since been pointed out that unless the time factor is also included no true comparison of degrees of efficiency can be made. We have overcome this valid objection by stopping certain experiments before the end of any fermentation, i.e., in all the cultures there was residual sugar when the final figures were obtained, and consequently the time factor was constant.

Experiment IX

A series of tubes containing 70 cc. of wort and various concentrations of NaCl were inoculated in the usual way. At the end of the experiment the following quantitative measurements were made: yeast crop, total CO_2 evolved and sugar utilized. From these figures these ratios were determined, $\frac{\text{yeast}}{CO_2}$, $\frac{\text{yeast}}{\text{sugar}}$ and $\frac{CO_2}{\text{sugar}}$. In table 9 the ratios from a number of experiments of this type are assembled. The results from experiments IX A, B show that there is a gradual increase in the fermenting efficiency of yeast, measured by sugar utilization and gas production, as the NaCl concentration is increased up to 4 to 5 per cent. Beyond this amount the efficiency falls. We are justified in accepting the qualitative significance of these results by those obtained in experiment IX C. In all the tubes fermentation was still in progress when the experiment was terminated. In other words the fermentation period was constant in all. During this unit of time the yeast in the 2 and 4 per cent tubes was more efficient than in the controls.

The ratio $\frac{CO_2}{\text{sugar}}$ changes show a rise from the control figure in

low salt concentrations when the total CO_2 output is stimulated, e.g., 1.0 and 2.0 per cent NaCl in experiment IX B. When this does not occur the ratio figure falls with increasing concentrations of salt. Before discussing the possible significance of this change we shall record an attempt to analyze more fully the $\frac{CO_2}{\text{sugar}}$ ratio for a normal fermentation and one containing NaCl.

TABLE 9

EXPERIMENT	NaCl	TIME OF FERMENTATION		SUGAR IN MEDIUM	$\frac{\text{SUGAR}}{\text{YEAST}}$	$\frac{CO_2}{\text{YEAST}}$	$\frac{CO_2}{\text{SUGAR}}$
	per cent	*hours*	*minutes*	*per cent*			
A	0	112	00	8.17	12.2	7.00	
	0				12.3	6.98	
	2				16.2	8.93	
	4				31.0	11.53	
	7				18.6	7.44	
	10				12.3	3.60	
B	0	107	00	8.0	16.6	5.2	0.310
	1				19.1	6.2	0.322
	2				23.6	8.3	0.350
	3				27.0	7.8	0.288
	4				31.1	8.4	0.270
	5				32.6	6.7	0.201
C	0	37	30	9.06	11.6	4.43	0.382
	0				12.4	4.82	0.389
	2				16.8	6.36	0.380
	4				18.2	5.43	0.298

Experiment X

Six tubes were inoculated with 1 cc. of culture. Three tubes were controls, and three contained 5.0 per cent NaCl. Tubes from the two groups were taken at intervals, and the CO_2 output and sugar utilization were determined.

In this experiment the culture tubes were equipped with aeration tubes to allow residual CO_2 to be replaced by air. The CO_2 was slowly washed into the absorption tubes just before the total CO_2 was determined for individual cultures. Small quantities

of medium were rapidly cleared for the sugar determinations. The experimental results and ratios are given in table 10. This type of experiment has been repeated several times, and it is quite definitely shown that the ratio $\frac{CO_2}{\text{sugar}}$ is not a constant throughout a normal yeast fermentation, but rises from about 0.350 to 0.450. It is necessary, therefore, to think not merely of a reaction or a group of coupled reactions rising and falling in intensity and giving uniform products, but rather of qualitative changes in addition to changes in activity. If we accept current views regarding the mechanism of yeast fermentation the ratio figures suggest an accumulation in the early stages of the fermen-

TABLE 10

CULTURE	TIME OF FERMENTATION		SUGAR UTILIZED	CO_2	$\frac{CO_2}{\text{SUGAR}}$
	hours	*minutes*	*grams*	*grams*	
Control	16	15	3.67	1.488	0.406
	21		6.27	2.587	0.413
	129	30	7.02	3.088	0.440
5.0 per cent NaCl	36	30	2.00	0.635	0.318
	61		3.05	1.023	0.335
	137		4.72	1.711	0.362

tation of one or more intermediates which precede the loss of CO_2 by pyruvic acid. It is possible to interpret the figures for cultures containing NaCl in one of two ways. The simplest interpretation is to regard them as relating essentially to an incomplete fermentation, and not qualitatively dissimilar from a control. The results from experiment X when plotted show two parallel curves which are similar in form, but the "salt" curve is below the control for similar degrees of sugar utilization. There is a suggestion, therefore, that NaCl accentuates an accumulation of labile products which is also characteristic of the early stages of a normal fermentation. Only a careful chemical examination of the products will enable us to say what is the precise significance of these changes in the ratios.

CONCLUSIONS

1. Wort containing NaCl up to a concentration of 10 per cent can be fermented by *S. cerevisiae*. The weight of the yeast crop obtained is reduced as the salt concentration is increased. We have no satisfactory evidence of growth stimulation by NaCl in this medium.

2. The lag-phase of the fermentation period is progressively lengthened by increasing concentrations of NaCl.

3. The influence of NaCl on the maximum rate of CO_2 production is dependent upon the size of the inoculum used and the salt concentration. Our experimental observations are summarized in table 11. We have observed similar effects on the total

TABLE 11

INOCULUM (DRY WEIGHT)	EFFECT OF NaCl ON MAXIMUM CO_2 RATE		
	0.5 per cent	1.0 to 1.5 per cent	1.5 to 10.0 per cent
grams			
0.004	Stimulation	Stimulation	Depression
0.020	Stimulation	Depression	Depression
0.400	No change	Depression	Depression

quantity of CO_2 produced. When NaCl is added at some point during an active fermentation low concentrations, i.e., 0.5 to 1.5 per cent, have no immediate stimulatory effect on CO_2 production. Higher salt concentrations under similar conditions depress the rate immediately.

We suggest that these differences are due to the following causes. When low concentrations of NaCl are brought into contact with actively fermenting cells the production rate of CO_2 is not stimulated, and as the salt concentration is increased an increased depression is observed. Yeast cells which are formed by vegetative growth in a medium containing the same low concentration of NaCl produce CO_2 more rapidly, i.e., following upon an increased lag-phase, and in greater quantities than in control cultures. The stimulating effect is not produced by an exposure of cells to the salt solution, but is intimately associated with cell

division. Therefore the observed effect of NaCl, e.g., 1.0 per cent, is determined by the relative numbers of old and new cells in the medium, and it may represent a balance between stimulation and depression. An indication of the possible difference in these numbers of cells is obtained by observing that 0.004 gram of yeast divides 7 times to become 0.400 gram, which is our average yeast crop, whereas an inoculum of 0.400 gram divides once to become 0.800 gram, the crop obtained when Brown's (1892; 1894; 1903) technique is followed.

4. The nitrogen content of yeast crops obtained from media containing NaCl, and inoculated with 0.002 to 0.040 gram of yeast increases until a concentration of approximately 6 per cent NaCl is used. From this point up to 10 per cent the N figure falls to that found for controls. When the inoculum used is a total crop of yeast, 0.400 gram, which ferments the medium with great rapidity and doubles in weight, the N figure changes qualitatively in a similar manner over the same range of salt concentration. Quantitatively the changes are much smaller. Yeast crops of this type exposed to the salted media for times equal to the length of normal fermentations do not show any greater changes in N content, indicating that the time factor is not important in this comparison. We consider that this quantitative difference between the two types of experiment is also due to the different effect of NaCl on existing cells and those formed by division in the experimental media. Recently it has been shown by Sohngen and Coolhaas (1924) that yeast cultures capable of fermenting galactose acquire this property by changes in the enzyme-complex of new cells formed in the medium containing the pentose. Acclimatization, therefore, is dependent upon a capacity to grow under the conditions of the experiment.

5. The ratio sugar fermented: yeast crop increases with the concentration of NaCl up to approximately 5.0 per cent. In higher concentrations the crops have a gradually diminishing efficiency. This relationship holds when the time factor for controls and salted cultures is constant, and its significance is increased in the light of a gradual prolongation of the lag-phase by these concentrations of salt. The changes is efficiency parallel those in N content.

6. As the concentration of NaCl increases (up to 2.0 per cent) the ratio CO_2:sugar utilized shows a gradual rise. In higher concentrations the ratio gradually falls to considerably below the control figure. We have found that during a normal wort fermentation this ratio rises, suggesting an accumulation of intermediates in the early stages. At present we cannot say whether the low ratios for high concentrations of NaCl represent incomplete normal fermentations, or whether they are qualitatively abnormal in their end-products.

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THE EFFECT OF HYDROXYL ION CONCENTRATION ON THE THERMAL DEATH RATE OF BACTERIUM COLI[1]

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INTRODUCTION

Strong acids and bases are known to be germicidal to bacteria. The growth limiting concentrations of hydrogen and hydroxyl ions have been determined for the more common organisms. Also, a number of investigations have been made on the effect of hydrogen ion concentration in accelerating the death rate of bacteria exposed to heat. This problem has been of particular interest to the canning industry. Less heat, it has been found, is necessary for complete sterilization of fruits and vegetables which are highly acid than for those which are more nearly neutral in reaction.

Less is known about the effect of alkalinity on the death rate of bacteria exposed to heat. Interest has arisen in this particular problem because of its bearing on the washing and sterilization of milk bottles and utensils. It would be of value in this connection to know just how important a factor the degree of alkalinity of the washing solution is.

It has been the purpose of this study to determine the magnitude of the effect of alkalinity in the destruction of bacteria. As a means of measuring the germicidal action of the hydroxyl ion,

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the effect of the pH of the medium of exposure on the thermal death rate of *Bacterium coli* has been studied.

HISTORICAL

As pointed out by Clark (1925), the results of many investigations have shown that the disinfectant action of an alkali is, in large measure, due to the hydroxyl ion concentration. A distinction must be made, however, between the more direct action of the hydroxyl ion concentration on bacterial cells, as in the case of strong bases, and the control of the effective state of a toxic compound by the concentration of hydroxyl ions.

Cohen (1922) showed how the viability of *Bacterium typhosum* in dilute buffer solutions was affected by various hydrogen ion concentrations. Cultures of *Bacterium typhosum* were planted in M/500 buffer solutions adjusted to pH values from 3.8 to 9.5 and these were incubated at 20°C. At all hydrogen ion concentrations decline in numbers occurred, but the least occurred at pH 5.0 and pH 5.4. At pH 3.8 the death rate was the greatest, the culture dying in six hours. At pH 9.5 the death rate was also rapid, the culture dying after twenty-four hours. The death rate, this experiment shows, is increased by a high pH value as well as by a low one; or, the death rate increases with an increase in hydrogen ion concentration below pH 5.0 and increases with an increase in hydroxyl ion concentration above pH 5.4.

The effect of the pH of the medium on the time necessary to destroy spores exposed to a high temperature has been studied by Bigelow and Estey (1920). They found that the thermal death rate was accelerated by an increased hydrogen ion concentration. At a temperature of 120°C. the time necessary to destroy spores of some thermophilic organisms in a medium of pH 4.6 was about one-fourth the time necessary for destruction in a medium of pH 6.0. The hydrogen ion concentration in this case caused a marked acceleration in the thermal death rate.

Chick and Martin (1912) have shown that an increase in the concentration of hydroxyl ions increases the denaturation rate of egg albumen in alkaline solutions. Although the coagulation of proteins of the type studied is probably different in many ways

from the destruction of bacterial cells, this work is no doubt a great help in understanding the process which takes place when the bacterial cell is destroyed by heat. It was found that, as the reaction proceeds, hydroxyl ions are continuously removed from solution.

Weiss (1921) showed that the thermal resistance of spores of *Clostridium botulinum* was lowered as the hydroxyl ion concentration was increased. At any given temperature, in a range between 95°C. and 120°C., the time necessary to destroy the spores at a pH of 8.2 was several times that necessary for destruction at a pH of 11.4.

From these investigations we would expect that the thermal death rate of *Bacterium coli* would be accelerated by an increased concentration of hydroxyl ions.

EXPERIMENTAL

Buffer mixtures

In preparation for carrying out experiments to determine the effect of hydroxyl ion concentration on the thermal death rate of *Bacterium coli*, the first step was the preparation of various buffer mixtures. During the course of the investigation, several different buffer mixtures were tried in an attempt to find a set of mixtures covering the alkaline range which would be free from any toxic effects caused by substances in the mixtures. Palitzsch's borax-boric acid mixtures, Sörensen's borate-sodium hydroxid mixtures, Ringer's secondary sodium phosphate-sodium hydroxid mixtures, Clark and Lubs' primary potassium phosphate-sodium hydroxid mixtures, were made up according to the directions given by Clark (1925). Since the mixtures were standardized electrometrically, no special precautions were taken to obtain materials of absolute purity. The sodium bicarbonate-sodium hydroxid mixtures were made up by adding M/10 sodium hydroxid to M/15 sodium bicarbonate till the desired pH values were obtained. In adjusting the peptone to various hydroxyl ion concentrations, N/10 sodium hydroxid was added to 2 per cent peptone.

Methods of determining pH

The electrometric method of determining the pH was used as the standard method. A Type K Leeds and Northrup potentiometer was used for this purpose. It was found desirable to use a colorimetric method also in this work. Preliminary adjustments of the exposure media and check determinations before and after experiments were made with a colorimetric method. For this purpose a La Motte Hydrogen Ion Testing Outfit, Model B, was used. This method is a refined spot test in which small, shallow glass vials are used as containers for the substances to be tested, and these are placed on a porcelain slab. By comparison with the colors of standard buffer solutions in other glass vials, to which indicator has been added, the pH of an unknown is readily determined. Check determinations with the electrometric method showed that the results were accurate to within 0.2 pH. The indicators used were as follows:

NAME OF INDICATOR	pH RANGE	CONCENTRATION AND SOLVENT
Brom thymol blue	6.0–7.6	As described by Clark (1925).
Phenol red	6.8–8.4	
Cresol red	7.2–8.8	
Thymol blue	8.0–9.6	
Cresol phthalein	8.2–9.8	
Thymolphthalein	9.3–10.5	0.04 gram in 50 cc. alcohol and 50 cc. water
Tropaeolin O	11.1–12.7	0.04 gram in 100 cc. water
Sodium indigo disulfonate	11.4–14.0	0.10 gram in 100 cc. water

History of culture of bacterium coli used

The culture used was obtained from Dr. Levine of the Iowa State College, and according to his classification was culture No. 24. It gave typical *Bact. coli* reactions: Positive methyl red test, negative Voges-Proskauer test, and did not ferment sucrose. It was carried on agar slants for three months previous to this experiment. During the investigation the culture was transferred daily in 1 per cent peptone solution. These cultures were grown at room temperature which remained about 25°C.

Preliminary experiments

Cultures of *Bacterium coli* grown in nutrient broth five days at room temperature were used in the first 2 experiments. The original broth culture was diluted 10 times with sterile water and this diluted culture was plated out on standard agar and counts made after forty-eight hours' incubation of 37°C. One cubic centimeter of this suspension was inoculated into each tube of exposure medium, there being 9 cc. of medium in each tube. The pH values of the different media ranged from 7 to 13. The number of bacteria in each cubic centimeter of the suspension in

TABLE 1

Effect of pH of exposure medium on destruction of Bacterium coli by heat

Culture grown in nutrient broth 5 days at 25°C.

pH suspension	Suspension exposed to temperature of 50°C.		Suspension exposed to temperature of 55°C.		Medium of exposure
	At start	After 10 minutes	At start	After 10 minutes	
	number per cc.	*number per cc.*	*number per cc.*	*number per cc.*	
7.0	53,000,000	10,200,000	71,000,000	4,000	Borax-boric acid
8.0	53,000,000	1,920,000	71,000,000	0	Borax-boric acid
9.0	53,000,000	0	71,000,000	0	Borax-boric acid
10.0	53,000,000	0	71,000,000	0	Borate-NaOH
11.0	53,000,000	0	71,000,000	0	Na_2HPO_4-NaOH
12.0	53,000,000	0	71,000,000	0	Na_2HPO_4-NaOH
13.0	53,000,000	0	71,000,000	0	NaOH

the exposure tubes at the start was computed from the count of the diluted culture. After 10 minutes' exposure 1 cc. of the suspension in the exposure tube was plated out on standard agar. One experiment was made at 50°C. and the other at 55°C.

The results are recorded in table 1. It can be seen from the table that at 50°C. a pH of 9 or greater was necessary for complete destruction in ten minutes, while at pH 7 about a fifth of the original number of bacteria survived after ten minutes' exposure. At 55°C., however, the number of bacteria was reduced to a small fraction of 1 per cent of the original number after ten minutes' exposure at pH 7, and the culture was completely destroyed at pH 8.

Other experiments were carried out for the purpose of determining the effects of different buffer mixtures on the viability of *Bacterium coli*. The same experimental procedure as described above was followed here also. In tables 2 to 7, inclusive, the results of this group of experiments are recorded. The discussion

TABLE 2

Comparison of effects of different buffer mixtures on thermal death rate of Bacterium coli

Temperature of exposure 50°C. Culture grown in 1 per cent peptone 3 days at 25°C.

TIME	MEDIUM OF EXPOSURE				
	Water	Borax-boric acid pH 7.0	Phosphate-NaOH pH 7.0	Phosphate-NaOH pH 8.2	Glycine-NaOH pH 8.4
	number per cc.	*number per cc.*	*number per cc.*	*number per cc.*	*number per cc.*
At start	7,800,000	7,800,000	7,800,000	6,600,000	6,600,000
After five minutes				270	504,000
After ten minutes	1,900,000	337,000	2,930,000	5	5,880
After twenty minutes				0	31

TABLE 3

Effect of concentration of hydroxyl ions in destruction of Bacterium coli

Temperature of exposure 25°C. Culture grown in 1 per cent peptone 3 days at 25°C.

pH OF SUSPENSION	SUSPENSION AT 25°C.		MEDIUM OF EXPOSURE
	At start	After 10 minutes	
	number per cc.	*number per cc.*	
8.0	1,510,000	1,270,000	KH_2PO_4-NaOH
9.1	1,510,000	730,000	KH_2PO_4-NaOH
10.0	1,510,000	25,000	$NaHCO_3$-NaOH
11.1	1,510,000	0	Na_2HPO_4-NaOH
12.0	1,510,000	0	Na_2HPO_4-NaOH
13.0	1,510,000	0	Na_2HPO_4-NaOH

of these results is taken up under Discussion, but one or two observations at present are probably advisable. In table 2 it can be seen that the mixture containing boric acid, which is a mild disinfectant, is more toxic than the phosphate mixture covering the same pH range. How much of this difference is

due to the concentration of salts and how much is due to the toxicity of certain ions and molecules is difficult to say. Other factors besides these two may enter also. The data are presented to show the necessity of a medium which exerts a constant effect

TABLE 4

Effect of concentration of hydroxyl ions in destruction of Bacterium coli

Temperature of exposure 40°C. Culture grown in 1 per cent peptone 3 days at 25°C.

pH of suspension	Suspension at 25°C.		Medium of exposure
	At start	After 10 minutes	
	number per cc.	*number per cc.*	
7.0	1,600,000	1,520,000	KH_2PO_4-NaOH
8.0	1,600,000	1,490,000	KH_2PO_4-NaOH
9.1	1,600,000	222,000	$NaHCO_3$-NaOH
10.0	1,600,000	400	Na_2HPO_4-NaOH
11.1	1,600,000	0	Na_2HPO_4-NaOH
12.0	1,600,000	0	Na_2HPO_4-NaOH
13.0	1,600,000	0	NaOH

TABLE 5

Effect of concentration of hydroxyl ions in destruction of Bacterium coli

Temperature of exposure 46°C. Culture grown in 1 per cent peptone 3 days at 25°C.

pH of suspension	Suspension at 46°C.		Medium of exposure
	At start	After 10 minutes	
	number per cc.	*number per cc.*	
8.9	14,500,000	1,944,000	Glycine
9.1	14,500,000	1,296,000	Glycine
9.3	14,500,000	307,000	Glycine
9.5	14,500,000	21,600	Glycine
9.7	14,500,000	7	Glycine
9.9	14,500,000	0	Glycine
10.9	14,500,000	0	Na_2HPO_4-NaOH

throughout the pH range of the experiment. Unless such a medium is used it is difficult to make comparisons between the effects of different concentrations of hydroxyl ions. In the data presented in tables 2 to 7, inclusive, it can be seen that the higher

the concentration of hydroxyl ions the more effective is the destruction of bacteria by heat. Within the same buffer mixture the effects of different pH values can be compared even though the death rates in different mixtures at the same pH are not the same.

TABLE 6

Effect of pH of medium of exposure on thermal death rate of Bacterium coli

Temperature of exposure 50°C. Culture grown in 1 per cent peptone 3 days at 25°C.

pH of suspension	Suspension at 50°C.				Medium of exposure
	At start	After 5 minutes	After 10 minutes	After 20 minutes	
	number per cc.	*number per cc.*	*number per cc.*	*number per cc.*	
7.0	6,600,000	49,700	2,300	190	KH_2PO_4-NaOH
7.5	6,600,000	13,300	190	40	KH_2PO_4-NaOH
7.9	6,600,000	210	0	0	KH_2PO_4-NaOH
8.2	6,600,000	270	5	0	KH_2PO_4-NaOH
8.4	6,600,000	504,000	5,880	31	Glycine
9.0	6,600,000	80	0	0	Glycine

TABLE 7

Effect of pH of medium of exposure on thermal death rate of Bacterium coli

Temperature of exposure 52°C. Culture grown in 1 per cent peptone 3 days at 25°C.

pH of suspension	Suspension at 52°C.		Medium of exposure
	At start	After 10 minutes	
	number per cc.	*number per cc.*	
6.96	2,930,000	0	KH_2PO_4-NaOH
7.89	2,930,000	0	KH_2PO_4-NaOH
8.15	2,930,000	0	KH_2PO_4-NaOH

Results with peptone as exposure medium

A 2 per cent solution of Difco peptone was prepared, several portions adjusted with M/10 sodium hydroxid to the desired pH values, and each mixture diluted with water so that the sum of the sodium hydroxid and water added in each case was a constant amount. Precipitates were filtered out after the solutions had been heated once. The pH changed during sterilization, the more

alkaline solutions decreasing as much as 0.4 pH. Since the pH values of the exposure media were determined immediately after each experiment, the final pH was the only one of importance.

TABLE 8

Effect of pH of medium of exposure on thermal death rate of Bacterium coli

Temperature of exposure 50°C. Medium of exposure—peptone solution.

pH OF SUSPENSION	SUSPENSION AT START	SUSPENSION AFTER				
		3 minutes	6 minutes	10 minutes	15 minutes	20 minutes
	number per cc.					
6.95	2,400,000	+	+	+	+	+
7.36	2,400,000	+	+	+	+	+
7.87	2,400,000	+	+	+	+	+
8.28	2,400,000	+	+	+	+	+
8.78	2,400,000	+	+	+	+	+
9.24	2,400,000	+	+	+	−	−
9.66	2,400,000	+	+	−	−	−
10.09	2,400,000	+	−	−	−	−
10.38	2,400,000	+	−	−	−	−

+, suspension not destroyed; −, suspension destroyed.

TABLE 9

Effect of pH of medium of exposure on thermal death rate of Bacterium coli

Temperature of exposure 52°C. Medium of exposure —peptone solution

pH OF SUSPENSION	SUSPENSION AT START	SUSPENSION AFTER				
		2½ minutes	5 minutes	10 minutes	15 minutes	20 minutes
	number per cc.					
7.05	2,380,000	+	+	+	+	+
7.44	2,380,000	+	+	+	+	−
7.94	2,380,000	+	+	+	−	−
8.34	2,380,000	+	+	−	−	−
8.78	2,380,000	+	−	−	−	−
9.22	2,380,000	+	−	−	−	−
9.69	2,380,000	+	−	−	−	−
10.11	2,380,000	−	−	−	−	−
10.28	2,380,000	−	−	−	−	−

+, suspension not destroyed; −, suspension destroyed.

Determinations, made before and after the disinfection tests in several experiments, showed that the pH did not change appreciably during the experiment.

Instead of making plate counts as in previous experiments, a different procedure was followed in this group of experiments. In order to determine when all the bacteria had been destroyed, 0.1 cc. samples of the bacterial suspension were inoculated into tubes of 1 per cent peptone. These samples were drawn from the exposure tubes at various intervals during the time of exposure. The inoculated tubes of 1 per cent peptone were then incubated at 37°C. for twenty-four hours. Turbidity of the peptone medium was recorded as an indication of growth. In this way

TABLE 10

Effect of pH of medium of exposure on thermal death rate of bacterium coli

Temperature of exposure 54°C. Medium of exposure—peptone solution

pH of suspension	Suspension at start	Suspension after				
		1 minute	2 minutes	5 minutes	10 minutes	20 minutes
	number per cc.					
7.0	3,920,000	+	+	+	+	−
7.4	3,920,000	+	+	+	+	−
7.9	3,920,000	+	+	+	−	−
8.3	3,920,000	+	+	+	−	−
8.6	3,920,000	+	+	−	−	−
9.0	3,920,000	+	+	−	−	−
9.5	3,920,000	+	+	−	−	−
9.8	3,920,000	+	−	−	−	−

+, suspension not destroyed; −, suspension destroyed.

times necessary to destroy all the bacteria at various pH values could be readily determined. Although the experimental error was large, many more points could be determined with this method than by the plating method. The results of these experiments are recorded in tables 8, 9, and 10. Curves plotted from these results are given under Discussion.

Determination of death rate of bacterium coli at 46°C. at different hydroxyl ion concentrations

Another series of experiments was carried out for the purpose of determining the death rate of *Bacterium coli* at various hydroxyl

ion concentrations. The procedure was altered somewhat from that used in any of the other experiments. A measuring flask (100 cc.) was used to hold the exposure medium. The flask was suspended in a constant temperature water bath. Five cubic centimeters of a three-day old culture, of which a sample was plated out at the time of inoculation, were inoculated into 50 cc. of the glycine-sodium hydroxid buffer mixture. The glycine-sodium hydroxid mixture as described by Clark (1925) was diluted to twice its volume. The exposure flask after the addition of the 5 cc. of the culture was shaken several times and was also shaken before the removal of each sample, but was at no time

TABLE 11

Death rate of Bacterium coli at pH 9.05

Temperature of exposure 46°C. Medium of exposure—glycine-NaOH mixture (diluted 1:1). Culture grown in 1 per cent peptone 3 days at 25°C.

TIME	SUSPENSION IN EXPOSURE FLASK AT 46°C.
minutes	*number per cc.*
0	27,400,000
2	28,300,000
4	33,300,000
8	28,500,000
12	29,000,000
16	25,500,000
20	19,100,000

taken from the water bath. At two-, four-, eight-, twelve-, sixteen-, and twenty-minute intervals 1.0 cc. samples were drawn and plated out on standard agar in duplicate. The plates were incubated at 37°C. for forty-eight hours.

From the data recorded in tables 11 to 13, inclusive, it is possible to determine the rate of death of *Bacterium coli* at 46°C. at 3 different hydroxyl ion concentrations. At pH 9.05 the decline in numbers was very slight even after twenty minutes' exposure. At a pH of 9.34 there was a significant increase in the death rate, and at a pH of 9.89 the decline in numbers was quite rapid. At the end of sixteen minutes' exposure at pH 9.89 all the bacteria

had been destroyed. The original concentrations of bacteria in all 3 experiments were approximately the same.

In tables 11 to 13, inclusive, one other thing of interest should be noted. If the logarithm of the number of survivors is plotted against time in minutes, a curve and not a straight line is obtained

TABLE 12

Death rate of Bacterium coli at pH 9.34

Temperature of exposure 46°C. Medium of exposure—glycine-NaOH mixture (diluted 1:1). Culture grown in 1 per cent peptone 3 days at 25°C.

TIME	SUSPENSION IN EXPOSURE FLASK AT 46°C.
minutes	*number per cc.*
0	30,000,000
2	28,200,000
4	28,200,000
8	11,500,000
12	4,940,00
16	610,000
20	90,000

TABLE 13

Death rate of Bacterium coli at pH 9.89

Temperature of exposure 46°C. Medium of exposure—glycine-NaOH mixture (diluted 1:1). Culture grown in 1 per cent peptone 3 days at 25°C.

TIME	SUSPENSION IN EXPOSURE FLASK AT 46°C.
minutes	*number per cc.*
0	34,300,000
2	15,800,000
4	1,160,000
8	9,000
12	100
16	0
20	0

in each instance. This fact shows that the death rate is not logarithmic. Velocity constants calculated by using the equation for the first order chemical reaction increased toward the end of the disinfection process. In these cases the rates of disinfection could hardly be measured satisfactorily by the velocity constants.

DISCUSSION OF RESULTS

pH-temperature relation

Figure 1 shows graphically the relation between the hydroxyl ion concentration and temperature in influencing the thermal death rate of *Bacterium coli*. The points plotted on this graph were taken from various experiments (see tables 3, 4, 5, 6, and 7)

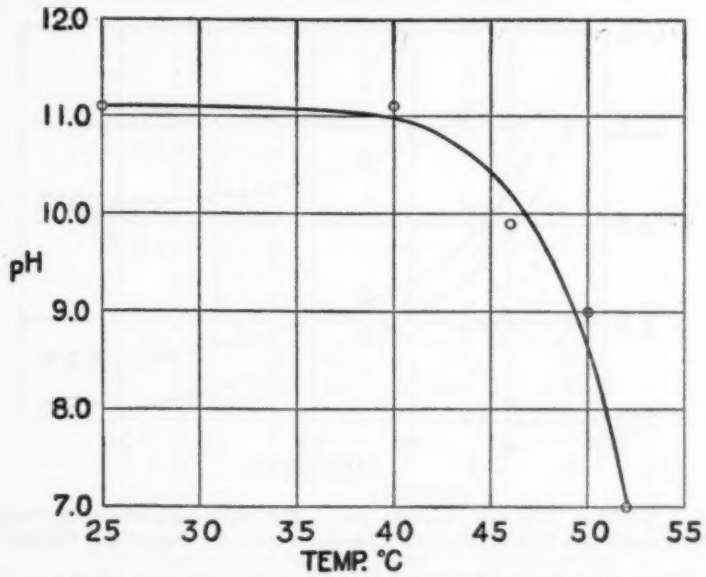

FIG. 1. pH-TEMPERATURE RELATION, SHOWING HYDROXYL ION CONCENTRATION NECESSARY FOR DESTRUCTION OF BACTERIUM COLI AT VARIOUS TEMPERATURES

Time of exposure ten minutes. Data from tables 3 to 7, inclusive

where the time of exposure was ten minutes. The points at 25° and 40° were obtained with secondary sodium phosphate buffer mixture as the medium of exposure; the points at 46° and 50° were obtained with the glycine buffer as the medium of exposure; and the point at 52° was obtained by the use of the primary potassium phosphate mixture. As has been shown above, the primary potassium phosphate mixture at approximately the same pH was

much more effective in the destruction of *Bacterium coli* than was the glycine mixture. Probably if the phosphate buffer had been diluted so as to decrease its buffer value, or if a more favorable medium such as glycine had been used, the point determined by a pH value of 7 would have fallen at a higher temperature. This would have made the curve slope more gradual at the higher temperature. However, the general relation is shown by the

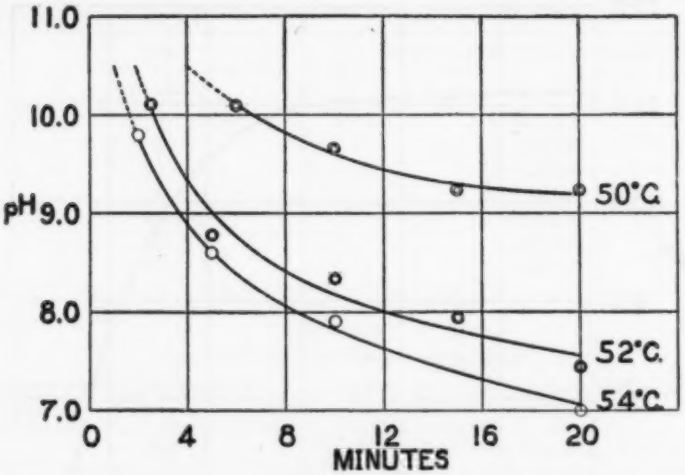

FIG. 2. pH-TIME RELATION, CURVES FOR 3 TEMPERATURES, SHOWING TIMES NECESSARY FOR DESTRUCTION OF BACTERIUM COLI AT VARIOUS pH VALUES

Medium of exposure: Peptone solution. Data obtained from tables 8 to 10, inclusive.

curve as it stands. Any point on the curve represents conditions which are destructive to *Bacterium coli* when the time of exposure is ten minutes.

pH-time relation

From the results of the experiments in which peptone, adjusted to the various pH values, was used as the medium of exposure, the curves in figure 2 were plotted; pH values on the ordinates, time in minutes on the abscissas. (For data see tables 8 to 10,

inclusive.) As is shown by the curves, when the temperature is decreased a higher pH value is necessary for destruction in a given length of time of exposure.

Temperature-time relation

From the curves in figure 2 the temperature and the time necessary for complete destruction of the bacteria at a given pH may be obtained. As shown in figure 3 a series of curves is

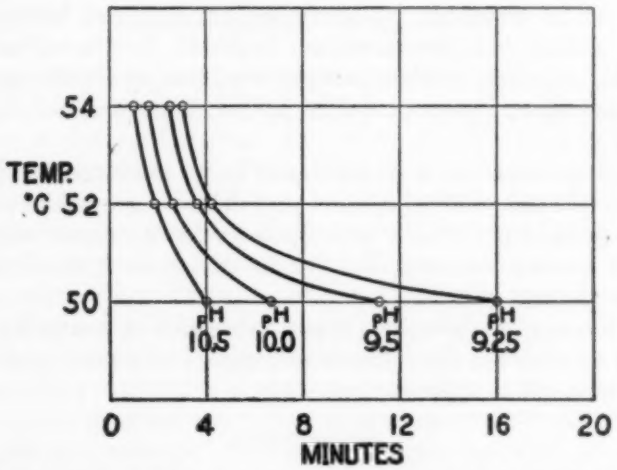

FIG. 3. TEMPERATURE-TIME RELATION, CURVES FOR 4 pH VALUES, SHOWING TIMES NECESSARY FOR DESTRUCTION OF BACTERIUM COLI AT VARIOUS TEMPERATURES

Data obtained from figure 2

obtained which show the relation of time to temperature at various pH values. The effect of the pH is quite evident again here, but one additional point is shown; namely, that a given difference in pH causes a greater difference in the thermal death time at 50° than at 54°. As the temperature is raised the curves tend to converge and approach zero time at high temperatures. As the temperature is lowered the curves flatten out, and the curves of the lower pH values extend to much longer times.

Similar relations for various organisms have been shown by Bigelow (1922). Weiss (1921) has determined similar curves with spores of *Clostridium botulinum* in which the temperature-time relation is shown at various pH values.

SUMMARY

1. Experiments have been made on the resistance of *Bacterium coli* to various conditions of temperature and hydroxyl ion concentration. Test mixtures used for this purpose were solutions of: Primary potassium phosphate-sodium hydroxid; borax-boric acid; sodium bicarbonate-sodium hydroxid; borate-sodium hydroxid; secondary sodium phosphate-sodium hydroxid; glycine-sodium hydroxid; peptone-sodium hydroxid; and sodium hydroxid alone.

2. It has been found that different buffer mixtures of approximately the same pH value exert very different germicidal effects. The effects of pH cannot be compared when 2 or more different buffer systems are used, but can be compared within a single buffer system.

3. Increase in the pH on the alkaline side of neutrality of a given solution has been shown to increase its power to destroy *Bacterium coli* at a given temperature.

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STUDIES IN THE CARBOHYDRATE METABOLISM OF PS. AERUGINOSA (B. PYOCYANEUS)[1]

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Many experimenters have investigated the nitrogen metabolism of *Ps. aeruginosa*. The subject is one to excite interest because of the very obvious and profound changes produced by this organism in the nitrogenous constituents of ordinary culture mediums. Change in the carbohydrate components of these mediums has not been so obvious and therefore little attention has been given to this subject. Many of the current texts fail to mention any fermentations at all by this organism and others dismiss the subject by saying that glucose is fermented. Even regarding this most readily attacked of all the sugars there are conflicting statements in the literature. Besson, Rangue and Senez (1918) reported the failure of this organism to ferment a considerable number of the common sugars including glucose. DeBord (1923) undoubtedly struck the real reason for this general confusion and lack of interest in the carbohydrate metabolism of *Ps. aeruginosa* when he reported that in 2 per cent peptone broth cultures containing 1 per cent glucose all of the sugar could be utilized without at any time producing an acid reaction. DeBord spoke of this as a different kind of carbohydrate fermentation.

More recently Sherwood, Johnson and Radotinsky (1926) have reported the results of fermentation tests on 22 strains of *Ps. aeruginosa*. A number of fermentable substances other than glucose were used by them. They found much variation in acid

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producing power among strains when litmus was employed as an indicator as well as by the pH determination method.

Considering the very active metabolism of nitrogenous substances by *Ps. aeruginosa*, even in the presence of glucose (Sears, 1916) it seemed probable that we were dealing here, not with a different kind of carbohydrate decomposition, but with a normal fermentation in which the acid end products of the reaction were masked by the products of nitrogen metabolism. Consequently, in order to avoid this masking action it should only be necessary to reduce the nitrogen metabolism to a minimum. It was shown

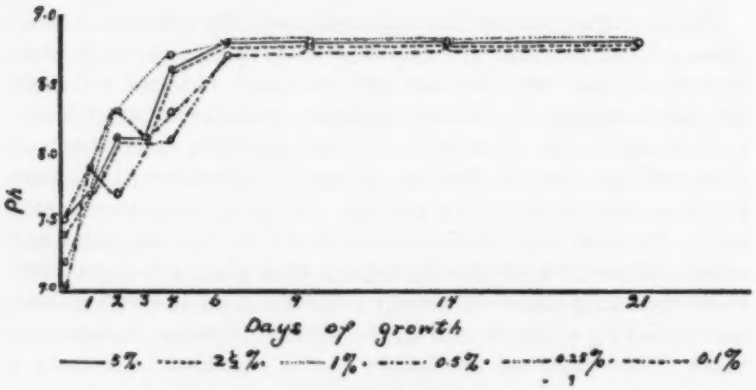

FIG. 1. PS. AERUGINOSA—pH IN PEPTONE SOLUTIONS OF VARYING CONCENTRATIONS

that this could be done with the ordinary peptone mediums by lessening the concentration of the peptone. Flasks containing 100 cc. of a bouillon composed of approximately 1 per cent glucose and concentrations of peptone varying from 0.1 to 5 per cent were inoculated with a young culture of *Ps. aeruginosa* and placed in an incubator at 37°C. At intervals samples were withdrawn and determinations of glucose, and pH were made. A series of control flasks without glucose were treated in the same way. Tables 1 and 2 give the data on this experiment. Figures 1 and 2 express the change in pH in the two sets of flasks and figure 3 shows the consumption of glucose.

By experiment with mediums of different H ion concentration it was shown that *Ps. aeruginosa* will grow in a peptone bouillon when the reaction lies between pH 4.0 and 8.8. It was observed that in the absence of glucose the reaction in all of the flasks be-

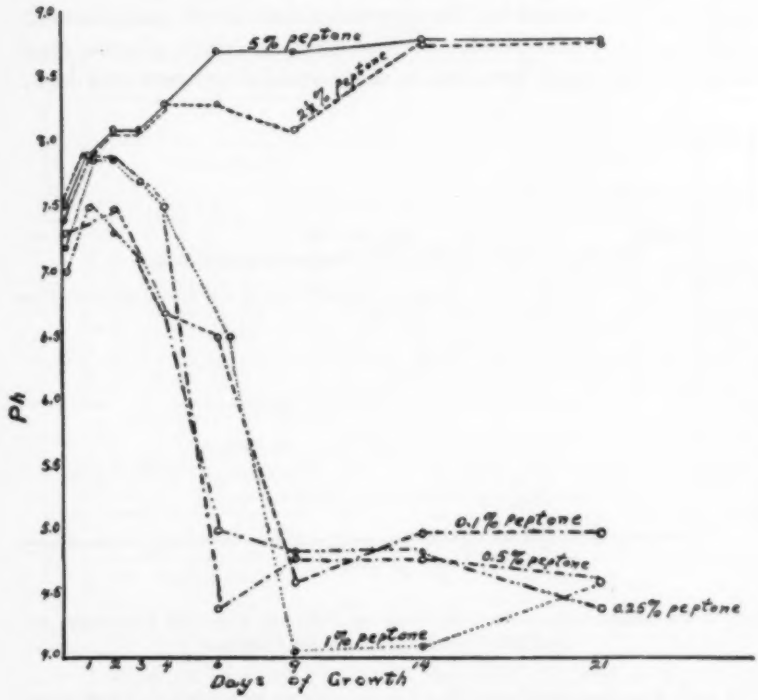

FIG. 2. PS. AERUGINOSA—pH CHANGE IN GLUCOSE PEPTONE SOLUTIONS HAVING VARYING CONCENTRATIONS OF PEPTONE

came continuously more alkaline until pH 8.8 was reached when it remained stationary. This limiting value was attained on the sixth day. In the glucose free mediums the curves for all the concentrations of peptone are seen to be practically identical. In the glucose bouillons on the other hand the curves differ widely. In the flasks having concentrations of 1 per cent or less

the pH drops and remains at a low point. In those having 2.5 and 5 per cent peptone the H ion concentration does not at any time rise above neutrality but falls rapidly to the limiting value of pH 8.8, where it remains.

Glucose consumption is, in general, inversely related to the rise in acidity, except for the surprising, and as yet unexplained, result in the 5 per cent peptone solution in which glucose consumption was much less than in the 1 and 2.5 per cent solutions.

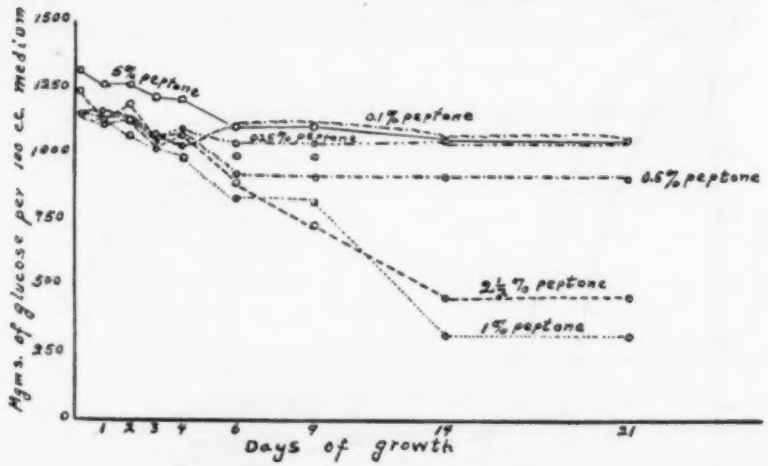

FIG. 3. PS. AERUGINOSA—GLUCOSE USED IN GLUCOSE PEPTONE SOLUTIONS OF VARYING CONCENTRATIONS OF PEPTONE

A set of experiments similar to the above in which 0.3 per cent meat extract was added to the medium and the peptone varied from 0.1 to 3 per cent gave results of practically the same import. As will be seen in table 3 the pH of the bouillons containing 2 and 3 per cent peptone rose from the beginning while that of the bouillons containing 1 per cent peptone had an initial fall followed by a rise to above neutrality. Practically all of the glucose was utilized in the cultures containing more than 1 per cent peptone.

DeBord stated that *B. subtilis* also belonged to the class of organisms which break down glucose without showing an acid

reaction. This has not proved to be true with the strain of *B. subtilis* used by us. Experiments exactly similar to the above carried out with this organism gave very different results. In glucose peptone solutions containing as much as 2.5 per cent peptone, even when highly buffered with a phosphate mixture as in

TABLE 1

Change in H ion concentration and buffer content in sugar free peptone solutions caused by the growth of Ps. aeruginosa (B. pyocyaneus)

CONCENTRATION OF PEPTONE	pH									BUFFER INDEX, pH 8.0 TO 5.0								
	Days of growth									Days of growth								
	0	1	2	3	4	6	9	14	21	0	1	2	3	4	6	9	14	21
per cent																		
5	7.5	7.7	8.1	8.1	8.6	8.8	8.8	8.8	8.8	3.25	4.35	5.75	5.60	5.65	5.45	5.55	4.50	4.89
2.5	7.4	7.7	8.1	8.1	8.6	8.8	8.8	8.8	8.8	1.65	2.28	2.98	3.22	3.50	3.95	2.48	2.98	2.55
1.0	7.2	7.7	8.3	8.1	8.7	8.7	8.8	8.8	8.8	0.63	1.00	1.25	1.47	1.50	1.75	1.53	1.12	0.98
0.5	7.5	7.9	8.3	8.1	8.3	8.7	8.8	8.8	8.8	0.25	0.70	0.78	0.85	0.85	0.93	0.78	0.80	0.55
0.25	7.0	7.9	7.7	8.1	8.1	8.7	8.8	8.8	8.8	0.13	0.35	0.43	0.63	0.53	0.50	0.43	0.45	0.26
0.1	7.3	7.9	7.7	8.1	8.1	8.7	8.8	8.8	8.8	0.13	0.25	0.25	0.33	0.38	0.63	0.22	0.22	0.17

TABLE 2

Change in H ion concentration and consumption of glucose by Ps. aeruginosa (B. pyocyaneus) in glucose peptone solutions

CONCENTRATION OF PEPTONE	pH								GLUCOSE, MILLIGRAM PER 100 CC. OF MEDIA							
	Days of growth								Days of growth							
	0	1	2	3	4	6	9	14		1	2	3	4	6	9	14
per cent																
5.0	7.5	7.9	8.1	8.1	8.3	8.7	8.7	8.8	1,325	1,260	1,260	1,200	1,200	1,100	1,100	1,050
2.5	7.4	7.9	8.1	8.1	8.3	8.3	8.1	8.8	1,240	1,130	1,180	1,070	1,070	890	730	460
1.0	7.2	7.9	7.9	7.7	7.5	6.5	4.0	4.0	1,150	1,130	1,070	1,010	990	830	820	310
0.5	7.5	7.9	7.9	7.7	7.5	4.4	4.8	4.8	1,150	1,120	1,140	1,010	1,060	910	910	910
0.25	7.0	7.5	7.3	7.1	6.7	5.0	4.8	4.8	1,150	1,120	1,100	1,040	1,070	1,040	1,040	1,040
0.1	7.3	7.5	7.5	7.1	6.7	6.5	4.6	5.0	1,170	1,170	1,130	1,080	1,030	1,100	1,100	1,050

DeBord's experiments, the pH fell as low as 5.6 and did not subsequently rise above that point. In the glucose free broth inoculated with *B. subtilis* the final reaction was pH 8.6. Glucose consumption by *B. subtilis* ceased when the concentration of the sugar was still more than 0.5 per cent.

The phenomenon called attention to by DeBord with respect to *Ps. aeruginosa* and *B. subtilis* has proved in our experiments to be true only of the first organism. Our experiments indicate that this phenomenon is definitely related to the vigorous nitrogen metabolism of *Ps. aeruginosa* which provides, in nitrogen rich mediums, sufficient alkaline substances to keep the acid products of sugar decomposition neutralized. In nitrogen poor mediums this is not accomplished and a marked rise in acidity results. It would seem that *Ps. aeruginosa* furnishes an exception to the rule first stated by Herter and often repeated by Kendall that fermentation always takes precedence over putrefaction. In this connection it is interesting to observe that glucose consumption

TABLE 3

Change in H ion concentration and consumption of glucose by Ps. aeruginosa in glucose, meat extract peptone bouillons having varying concentration of peptone

CONCENTRATION OF PEPTONE	pH						GLUCOSE					
	Days of growth						Days of growth					
	0	1	2	3	7	15	0	1	2	3	7	15
per cent												
3	6.7	7.9	7.9	7.8	8.1	8.6	1,190	980	970	800	270	0
2	6.9	7.6	7.4	7.5	7.5	8.6	970	910	660	620	360	0
1	6.9	7.9	6.3	5.1	5.2	7.2	980	860	810	470	0	0

(fig. 3) proceeds slowly during the first two days of growth while nitrogen metabolism, as recorded by several investigators (Sears, 1916; DeBord, 1923) takes place rapidly during this early period.

The active nitrogen metabolism of *Ps. aeruginosa* not only is capable of preventing the appearance of limiting acidity in its own cultures but when grown in symbiosis with more vigorously fermenting organisms exerts a decided effect upon the carbohydrate consumption of the latter. Table 4 and figure 4 show this effect upon *Esch. coli*. The last named organism, even in the higher concentrations of peptone, ferments glucose rapidly during the first days of growth. It soon, however, produces an acidity which completely stops further action on the sugar. Not more than 350 to 400 mgm. of glucose per 100 cc. of medium are

decomposed. When *Ps. aeruginosa* and *Esch. coli* are inoculated together we see a different result. The pH falls, to be sure, nearly to the same point as in coli cultures alone, but glucose consumption goes on, where the peptone content is 2 per cent or higher, practically to completion.

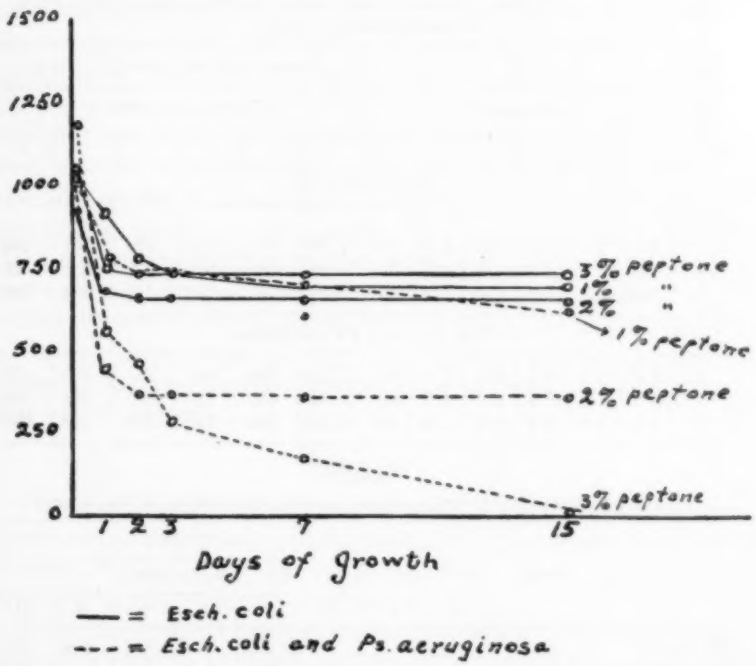

FIG. 4. ESCH. COLI AND PS. AERUGINOSA—GLUCOSE USED IN THE PRESENCE OF VARYING CONCENTRATIONS OF PEPTONE

The employment of the methods outlined above, namely, the reduction of the concentration of peptone in the medium, failed to show fermentation by *Ps. aeruginosa* of carbohydrates and alcohols other than glucose. As is seen in table 5, in a 0.5 per cent peptone solution containing 1 per cent of various fermentable substances the pH rises at once and remains above neutrality,

though in no case does it rise as high as in the same medium without sugar. Another test which fails to indicate decomposition of some of these sugars is that of symbiotic gas production described by Sears and Putnam (1923). If *Ps. aeruginosa* is

TABLE 4

Change in pH and glucose consumption by Esch. coli and by the symbiotic complex, Esch. coli-Ps. aeruginosa in glucose, meat extract bouillons containing varying concentrations of peptone

CONCENTRATION OF PEPTONE	pH						GLUCOSE, MILLIGRAM PER 100 CC. MEDIUM					
	Days of growth						Days of growth					
	0	1	2	3	7	15	0	1	2	3	7	15
	Esch. coli alone											
per cent												
3	6.7	4.7	4.7	4.6	4.7	4.6	1,050	750	740	740	740	740
2	6.9	5.0	4.9	4.8	4.8	4.6	930	690	670	670	660	660
1	6.5	4.6	4.6	4.4	4.4	4.4	1,030	860	780	740	700	700
	Esch. coli and *Ps. aeruginosa*											
3	6.7	4.8	4.7	4.8	4.8	4.8	1,190	560	460	290	170	00
2	6.9	4.9	4.6	4.6	4.6	4.6	1,050	450	370	370	360	360
1	6.8	4.6	4.6	4.6	4.6	4.6	1,030	780	740	740	700	640

TABLE 5

Change of pH in 0.5 per cent peptone solutions containing various sugars

SUGAR	pH					
	Days of growth					
	0	1	2	5	7	11
No sugar	7.2	8.0	8.3	8.6	8.8	8.8
Glycerol	7.0	7.9	8.1	8.3	8.6	8.6
Mannitol	6.9	7.7	7.9	8.1	8.0	8.0
Maltose	7.0	7.5	7.7	8.0	8.1	8.3
Sucrose	7.3	7.9	7.9	8.3	8.3	8.3

capable of attacking a given sugar without gas formation then growing this organism on mediums containing that sugar together with an organism not capable of fermenting the sugar but able to ferment glucose with gas should result in the formation of gas. All such tests were negative.

That a number of sugars and alcohols other than glucose are utilizable, under certain circumstances at least, by *Ps. aeruginosa* has been demonstrated by Goris and Liot (1923) and Liot (1923) and appears also from the following experiments of ours. It has long been known that *Ps. aeruginosa* is capable of utilizing ammonium tartrate as its nitrogen source if an independent source of carbon is available but that it cannot procure both elements from this compound (Fischer, 1900). A medium was prepared consisting of a 1 per cent aqueous solution of tartrate containing 0.2 per cent dipotassium phosphate, 0.1 per cent magnesium sulphate and 0.01 per cent calcium chloride. Five different strains of *Ps. aeruginosa* were tested for growth in this medium and also in the same medium containing 1 per cent of various sugars and alcohols. The results were profuse growths of all strains when the medium contained glucose, glycerol, mannitol, levulose, galactose, mannose, sorbitol or maltose, no growth or doubtful growth of all strains when the medium contained lactose or sucrose or no sugar at all. The strains reacted differently toward xylose and inositol, two utilizing the former sparingly and only one the latter.

SUMMARY

Ps. aeruginosa produces an acid reaction in glucose peptone mediums and in glucose peptone meat extract mediums when the nitrogen content of the medium is kept low. Under these conditions only a small amount of glucose is used.

When the nitrogen content is high as represented by 2 per cent or more of peptone no acid reaction is produced but large amounts of glucose are utilized.

When other sugars are used in place of glucose an acid reaction is not produced even when the nitrogen content is low. Many of these sugars can be utilized by *Ps. aeruginosa* however as may be shown by use of an ammonium tartrate mineral salt medium in which the organism will not grow when sugar free. With the addition of a number of common laboratory sugars good growth is obtained in such a medium.

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It is characteristic of Science and Progress that they continually open new fields to our visions.—PASTEUR.

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ELECTROPHORESIS OF DIPHTHERIA BACILLI

I. SOME RELATIONS BETWEEN THE VIRULENCE, TOXIGENICITY AND ELECTROPHORETIC MOBILITIES OF DIPHTHERIA BACILLI

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I. INTRODUCTORY

The bacteriology of diphtheria has often been regarded as a subject upon which further work would be unprofitable. This opinion was formed, no doubt, from the brilliant and decisive studies of Loeffler (1884; 1887), and of those who followed within a decade and developed the fundamentals of both theory and method in the bacteriology and toxicology of this disease. Loeffler's staining method is to this day preferred by many workers. The coagulated serum medium devised by him is regarded as superior to any culture medium since advocated for the rapid growth of diphtheria bacilli. He also emphasized the importance of distinguishing the true virulent diphtheria bacilli from the pseudo-diphtheria bacilli by testing the virulence on guinea pigs and not relying altogether on the microscopical diagnosis. In reading Loeffler's classical papers one is struck by the fact that the enormous amount of work done on this subject since then [with the exception of the discovery of diphtheria toxin by Roux and Yersin (1888), and antitoxin by Behring (1890)] has been largely along the lines layed down by him in his publications of 1884 and 1887.

The voluminous literature appearing since these early researches has dealt with biochemical characteristics, cultivation, morphology and staining, virulence and toxin production, the major portion of the work (modifications of Loeffler's methods)

having been directed towards the development of aids for the clinician in his special province.

The production of a potent toxin in order to make satisfactory antitoxin has also been the objective of intensive study. The technique of toxin production is, for the most part, purely empirical in character. While many interesting investigations have been made, very few of them have shed any real light on the mechanisms of the process or have demonstrated the true variables involved.

During the past few years Falk and his associates (Falk, Gussin and Jacobson, 1925; Falk, Jacobson and Gussin, 1925a and b; Falk and Jacobson, 1925, 1926a and b), have presented at considerable length analysis of certain problems concerned with the virulence of bacteria. They succeeded in substituting for the usual animal technique in testing for virulence, physio-chemical measurements which enabled them to conduct new and unusual experiments. Although it has long been known that agglutinability or stability of suspension in the case of virulent or aggressive bacteria is correlated with virulence to animals, the mediating mechanism was unknown until it was found that alterations in the electrophoretic potential of bacteria were associated with these reactions. The authors mentioned above then designed experiments to determine the relations between these electrophoretic potential differences and the virulence of bacteria. During the course of their work on pneumococci they found that there are significant parallelisms, direct or inverse, between electrophoretic potential, virulence for white mice and fatality for man, agglutinability, colony formation, phagocytic reactions or opsonic indices, sodium oleate or bile solubility and other characteristics of these bacteria. For pneumococci of the types I, II, III and IV it was found that the P.D. is higher, the greater the virulence for white mice, and *vice versa*. For variant strains of type I and for R and S variants the same relations were demonstrated. The highest potentials (mobilities) were found on type III pneumococci. The sequence of decreasing potential was shown to be III, I, II, IV,—which is identical with that for decreasing virulence for white mice and also for the probable

sequence for fatality rates in pneumococcus pneumonias of man. It was found for pneumococci of types II, III, and IV, that cultures isolated from fatal cases of acute lobar pneumonia showed higher electrophoretic potentials than those obtained from non-fatal cases. The reverse was observed for cultures of type I. In the work on phagocytosis the authors were able to show that the presence of acids, alkalies, salts and other reagents modified the electrophoretic velocity and the extensiveness of phagocytic reactions. For example, they found that if the P.D. on pneumococci be reduced by such a salt as $La(NO_3)_3$, the phagocytosis is increased proportionately. If the P.D. be increased by Na oleate, the phagocytosis is reduced. The same relationship was demonstrated when the cocci were treated first with $La(NO_3)_3$, washed, and then treated with Na oleate, and when the procedure was reversed.

Thus from the many types of experimentation to which the pneumococci especially lend themselves, there was developed a new avenue of approach to the study of virulence of other pathogenic bacteria.

The work here reported was undertaken with the hope that by the use of similar physical measurements a presumptive analysis of virulence of the diphtheria bacilli might be possible. For a more complete discussion of the theory of virulence, cf. Falk, (1928).

II. VIRULENCE TESTS FOR THE IDENTIFICATION OF C. DIPHTHERIAE

Investigations of the biochemical characteristics of corynebacteria have not led to the establishment of any certain differentiating properties with which virulence may be correlated. Differences in morphology have not been of great aid as criteria of virulence. The animal test for virulence has been regarded as a final identification of corynebacteria suspected of being virulent diphtheria bacilli. The animal test, however, is conducted in so many ways that the method should always be stated to show whether "virulence" or "toxigenicity" is operative. "Virulence," as defined by Theobald Smith, signifies the behavior of diphtheria

bacilli towards living tissue; whereas "toxigenicity" is concerned with the capacity to produce toxin. The difference between

CHART 1

	"VIRULENCE" TESTS FOR GUINEA PIGS	EXPRESSION OF		CRITICISM	TIME FOR IDENTIFICATION
		Virulence	Toxigenicity		
					days
1	48-hour whole broth 1 to 4 cc. subcutaneously	+	+	None	4–6
2	48-hour filtered broth, 1 to 4 cc. subcutaneously	−	+	Strains of low toxigenicity missed. Lost in filter	4–6
3	48-hour broth centrifugated broth. Subcutaneously	−	+	Strains of low toxigenicity missed	4–6
4	Bacilli from centrifugated broth. Subcutaneously	+	−	Low virulence and virulent pseudo-diphtheria	4–6
5	Bacilli from Loeffler or glycerol agar slants	+	−	Low virulence and virulent pseudo-diphtheria	3–6
6	Filtered broth. Intracutaneously	−	+	Recognition of lesion, dilution factor low toxigenicity	3
7	Bacilli from Loeffler. Intracutaneously	+	−	Recognition of lesion, and virulent pseudo-diphtheria	2+
8	Whole broth cultures. Intracutaneously	+	+	Recognition of lesion, and virulent pseudo-diphtheria	3
9	Crude culture (broth). Subcutaneously	+	+	Contaminating organisms and low virulence	4
10	Fragments of membrane into guinea pig by incision into skin	+	−	Contaminating organisms and low virulence	4
11	Intracutaneous inoculation	+	−	Contaminating organisms and low virulence	2+
12	Centrifugated crude broth culture. Subcutaneously or intracutaneously	+	−	anti-biosis	4–6

virulence and toxigenicity has been brought out experimentally by Martin (1898), Arkwright (1911), Cary (1917), Barratt (1923) and others. Also in the course of this report it will be

shown that the majority of true diphtheria bacilli vary in their toxigenicity and virulence—some being highly toxigenic *in vitro* and almost avirulent *in vivo*, and *vice versa*. Cary pointed out that if the Park No. 8 strain were tested for virulence by injecting salt solution suspensions into guinea pigs, it would usually be regarded from the results of such an experiment as an avirulent culture. He found that in one case the growth from 2.4 Loeffler medium slant cultures, washed off in salt solution, did not produce fatal results in the unprotected guinea pig.

A summary and criticism of the methods for testing "virulence" now in use in public health laboratories is shown in chart 1.

The test for virulence used in our studies was conducted as follows: 50 cc. amounts of veal infusion (1 pound veal to 1 liter of distilled water, infused overnight in ice-box) + 2 per cent Witte's peptone − salt-free broth + 0.1 per cent glucose were adjusted to pH 7.4, inoculated, and incubated for forty-eight hours at 37°C. Each broth culture was then injected subcutaneously into 250-gram guinea pigs. Failure of 4 cc. of the whole broth culture to produce death in seven days with typical necropsy findings was considered sufficient ground to call a strain avirulent and atoxigenic. Our reasons for this criterion of virulence have already been stated.

It is important to recall at this point that the qualitative distinction between virulent (toxigenic) and avirulent (non-toxigenic) corynebacteria rests upon a spurious basis. It is commonly observed that if virulence be measured quantitatively in terms of the minimal fatal quantity of culture, filtrate, etc., the highly virulent strains grade into the slightly virulent or avirulent. When it is arbitrarily assumed that a strain is virulent if a particular quantity of culture, filtrate, etc., kills, and is avirulent if that quantity fails to kill, a qualitative, discontinuous demarcation is established, not by characteristics of the series of cultures being tested, but by the nature of the criterion (death or survival) used. In all of our work we have found no sharp line of demarcation between virulent and avirulent strains of diphtheria bacilli, although such a line may exist between true diphtheria bacilli and other groups among the corynebacteria.

III. SOURCES OF CULTURES

"Pill boxes" containing Loeffler's medium which had been inoculated with material from suspected cases of diphtheria and suspected carriers were supplied to us by the Chicago Department of Health. Some additional strains were sent to us by R. L. Laybourn from school outbreaks in Missouri and Iowa. These cultures were streaked on blood agar plates and incubated for eighteen hours at 37°C. Well isolated colonies were replated on sheep's blood agar after microscopical examination had been made to determine purity. After eighteen hours' incubation, well isolated colonies were again chosen, stained preparations examined and transfers made to blood agar slants. After several transfers on blood agar slants all cultures grew readily when inoculated on glycerol agar slants containing 1 per cent beef serum. In some instances broth was made sugar-free and buffered with 0.3 per cent Na_2HPO_4, the final reaction being pH 7.2 before inoculation with pure cultures. Changes in methods are indicated in the specific protocols of the experiments. Sugar-free broth was prepared by inoculating the medium with *Bact. coli*, incubating twenty-four hours at 37°C. and sterilizing by filtration through tested Mandler filters. It was necessary to free the medium of the *Bact. coli* cells because of their interference in microscopic cataphoresis experiments.

Three hundred and eighty strains of corynebacteria were used in the studies reported here. The source and characteristics of some of the strains will be listed in the tables that follow. Most of the cultures had been obtained from children ranging in ages from six months to sixteen years.

IV. FERMENTATION REACTIONS

The fermentation formula for *C. diphtheriae* has been well established by Barratt (1923). It has not been possible to correlate virulence or toxigenicity with fermentative powers of diphtheria bacilli. In almost any large collection of avirulent strains obtained in surveys, i.e., throat cultures, certain of these races will be found to give biochemical reactions identical with those of toxigenic bacilli.

A variety of "sugars" was available and it was thought desirable to test the fermentative powers of a number of avirulent and virulent strains. Veal infusion broth was rendered sugar-free, adjusted to pH 7.4, tinted with brom cresol purple, tubed and sterilized. The test substances were dissolved in distilled water and sterilized by filtration through tested Mandler filters. Sufficient sugar solution was added aseptically to each tube, to give a final concentration of 0.5 to 1.0 per cent.

Forty-five virulent and five avirulent (not pseudo-diphtheria) strains of diphtheria bacilli were indistinguishable when tested on the following substances: fucose, d-arabinose, a-mannoheptose (perseite), a-glucoheptose, cellose, melibiose, trehalose, melezitose, rhamnose, xylose, levulose, glucose, galactose, mannose, sucrose, lactose, maltose, raffinose, inulin, starch, glycogen, dextrin, dulcitol, erythritol, d-mannitol, adonitol, d-arabitol, l-arabitol, glycerol, arbutin, alpha-methyl glucoside, salicin, amygdalin, mannonic acid lactone, gulonic acid lactone, and glucosamine hydrochloride. Of these test substances glucose, levulose, galactose, maltose, dextrin and glycerol were fermented alike by the 50 strains. Acid was not produced from any of the other test substances except glucosamine hydrochloride. Five virulent and one avirulent culture failed to ferment this substance.

V. THEORETICAL BASIS FOR ELECTROPHORESIS STUDIES

It was shown by Donnan (1911) that in certain cases the potential difference (P.D.) between two solutions of an electrolyte, separated by a membrane which is impermeable to the electrolyte but permeable to the solvent, can be calculated in the following manner: Suppose the solutions (1) and (2)

$$\begin{array}{lll} (1) & K^+ & K^+ \quad (2) \\ & A^- & A^- \\ & C_1, \pi_1 & C_2, \pi_2 \end{array}$$

of the electrolyte KA are separated by a membrane which does not permit the salt KA to pass, though freely permeable to other salts with the same cation K. Donnan and Green (1914) as-

cribe the P.D. set up at the membrane as due to the tendency of the K^+ ions to equalize their concentrations.

If we assume the permeable ions to obey the laws of ideal solutions, then

$$\pi_2 - \pi_1 = \frac{RT}{nF} \log \frac{C_1}{C_2},$$

where C_1, C_2 are the molar concentrations of the K^+ ions, n their valency, R the gas constant, F the quantity of electricity associated with the gramme-equivalent of ionic matter, and π_1, π_2 positive potentials of the solutions. For univalent ions and 18° this reduces to $\pi_2 - \pi_1 = 0.058 \cdot \log\left(\frac{C_1}{C_2}\right)$ volts.

As the numerator decreases and the denominator increases in the molar concentrations $\left(\frac{C_1}{C_2}\right)$ of K ions, the P.D. obviously decreases. If we suppose that the membrane is not absolutely but only partly impermeable to the electrolyte, so that equilibrium across it can be attained only in a longer or shorter period of time, it follows that the greater the permeability the lower the potential difference will be after any given time interval. Loeb (1922) showed that the Donnan effect might alone determine the depressing effect of electrolytes on the P.D. of suspended particles of gelatin. Winslow, Falk and Caulfield (1923) observed that neutral salts depressed mobilities of bacterial cells, calcium salts being more effective than sodium salts of the same concentration. They observed further that, as demanded by the Donnan theory, heat killed bacteria exhibit essentially the same curve of velocity as that of the living cells. Their studies led them to state:

> Professor Donnan and other investigators have clearly understood the importance of applying the concept of membrane equilibria in the elucidation of physiological phenomena. Our findings add to the numerous indications favoring this view and emphasize the importance of further study of membrane equilibria in bacterial suspensions.

Postulating that the differences between strains of diphtheria bacilli which do, and those which do not, excrete toxin are de-

pendent not upon differences in capacities to produce but to excrete toxin, it was anticipated that electrophoresis measurements would show different P.D. values for toxigenic and non-toxigenic strains. The postulate assumes that all strains can produce toxin, but only those with surfaces that are relatively permeable to toxin will excrete it. Hence, for toxin excreting strains it should be found that $\frac{C_1}{C_2}$ (where C_1 and C_2 are now concentrations of toxin inside and outside the cell) will have a relatively smaller value than for non-excreting strains; and the measurable P.D. should be lower accordingly.[1]

VI. MEASUREMENT OF ELECTROPHORETIC MOBILITIES

The electrophoretic (cataphoretic) potentials on corynebacteria were measured by the method described and used by Falk, Gussin and Jacobson (1925a). For reasons discussed by Winslow, Falk and Caulfield (1923), results are expressed in terms of observed electrophoretic velocities (μ per second). Ten measurements of velocity (five with one and five with reversed orientation of the electrical field) were made at stationary layers [*V.* (133) (497)] at room temperature in the same medium in which the bacteria were grown except where another medium is specified later. P.D. in observed velocities (μ per second) may be converted into millivolts by multiplying by the factor 1.3. The same methods were used in measuring velocities of cells in distilled water. Hydrogen ion concentrations were determined colorimetrically.

VII. ELECTROPHORETIC MOBILITY AND VIRULENCE OF FORTY-EIGHT-HOUR WHOLE BROTH CULTURES OF DIPHTHERIA BACILLI

In table 1 we present the results of a number of experiments performed on purified cultures of corynebacteria. The data have

[1] It is clearly recognized that although the investigations described here were planned on the hypotheses outlined, the validity of the experimental findings is in no wise dependent upon the validity of the theoretical treatment. Statements made here to describe a working hypothesis are not intended to be a commitment to the theory that P.D. or charge necessarily has origin in a membrane equilibrium.

TABLE 1

Tabulation of strains showing parallelism between virulence and electrophoretic potentials

CULTURE	P.D. 48-HOUR VEAL BROTH	VIRULENCE*	FERMENTATION		SOURCE
			Sucrose	Glucose	
	μ/second				
A10	12.1	−	+	+	Suspected
A3	11.7	−	+	+	Suspected
A4	10.0	−	−	−	Normal
10	9.4	−	−	−	Normal
2A	9.4	−	−	−	Suspected
6	9.2	−	−	−	Suspected
A27	9.2	−	−	−	Normal
2C	8.9	−	−	−	Suspected
44	8.5	−	−	−	Carrier
W730	8.3	−	−	−	Normal
2	7.9	−	−	−	Suspected
34	6.2	−	−	−	Rhinitis
39	6.2	−	−	+	Carrier
13–2	5.8	−	−	+	Normal
13–1	5.7	−	−	+	Normal
32	5.9	−	−	+	Carrier
14	5.6	−	−	+	Control
41	4.5	−	−	+	Carrier
33	4.4	−	−	+	Carrier
17	4.2	+	−	+	Suspected
9	4.1	+	−	+	Terminal
7	4.0	+	−	+	Terminal
29	4.0	+	−	+	Suspected
8	3.8	+	−	+	Carrier
46	3.8	+	−	+	Carrier
A22	3.7	+	−	+	Suspected
19	3.7	+	−	+	Clinical
B1	3.6	+	−	+	Unknown
12	3.6	+	−	+	Carrier
15	3.6	+	−	+	Case
1	3.6	+	−	+	Suspected
25	3.6	+	−	+	Terminal
5	3.5	+	−	+	Suspected
28	3.4	+	−	+	Suspected
27	3.4	+	−	+	Suspected
20	3.4	+	−	+	Suspected
36	3.4	+	−	+	Carrier
A2	3.4	+	−	+	Suspected
26	3.2	+	−	+	Suspected

TABLE 1—*Continued*

CULTURE	P.D. 48-HOUR VEAL BROTH	VIRULENCE*	FERMENTATION		SOURCE
			Sucrose	Glucose	
	μ/second				
48	3.2	+	−	+	Suspected
49	3.1	+	−	+	Suspected
31	3.1	+	−	+	Terminal
A26	3.0	+	−	+	Carrier
47	2.9	+	−	+	Suspected
23	2.8	+	−	+	Suspected
50	2.8	+	−	+	Terminal
A21	2.6	+	−	+	Clinical
A6	2.3	+	−	+	Suspected
Park No. 8	1.0	+	−	+	Laboratory stock

* Virulence is here used to mean ability to produce death in unprotected guinea pigs following subcutaneous injection of 1 cc. amounts of veal infusion cultures (unfiltered) which have been incubated two days at 37°C. The result may be an expression of the virulence of a culture together with the toxigenicity.

been arranged with the culture showing the highest mobility (in micra per second) first and the culture with lowest mobility last. Measurements of mobility on forty-eight-hour veal broth cultures of toxigenic strains gave values of 4.2 to 1.0 (μ per second) with an average of 3.4 (μ per second). Measurements of mobilities on avirulent (non-toxigenic) strains which fermented sucrose gave higher values (12.1 μ per second). Non-fermenting avirulent cultures (*C. hoffmanni*) gave values of 7.9 to 10.0 (μ per second). Strains with P.D. of intermediate values, 4.4 to 6.2 (μ per second), are according to morphological and biochemical characteristics typical diphtheria bacilli but are not virulent or toxigenic.

VIII. INFLUENCE OF pH ON MOBILITIES OF DIPHTHERIA BACILLI IN FORTY-EIGHT HOUR BROTH CULTURES

The demonstration that an inverse relationship exists between P.D. and virulence of whole broth cultures, as appears from the data in table 1, is open to criticism on the ground that the majority of the cultures showing low mobilities were also glucose fermenters. This would imply that the acidity of fermentation was

marked enough (pH 5.8 to 6.6) to lessen electrophoretic velocities. Inasmuch as potential difference (mobility) is known to be a function of pH, the experiments shown in table 2 were performed to verify the assumption that the acidity produced was not sufficient to account for the differences in observed velocities. Measurements were made on forty-eight-hour cultures grown in sugar-free, buffered broth and in veal infusion broth. Further proof that our original interpretation was correct is shown later.

TABLE 2

Comparison of mobilities in sugar-free, buffered broth (0.3 per cent Na_2HPO_4) and in untreated veal infusion broth. Initial reaction, pH 7.2

CULTURE	P.D. SUGAR-FREE	pH	P.D. VEAL INFUSION	pH	VIRULENCE	FERMENTATION
	μ/second		μ/second			
6	10.6	7.2	9.7	6.6	−	−
2A	8.5	7.4	8.5	6.6	−	−
10	6.8	7.6+	8.5	7.6	−	−
32	5.9	7.2	6.6	5.8	−	+
14	5.6	7.2	5.6	5.8	−	−
27	3.7	7.4	3.4	5.8	+	+
28	5.5	7.2	3.4	5.8	+	+
46	3.8	7.2	3.5	5.8	+	+
12	3.7	7.2	3.6	5.8	+	+
25	3.7	7.2	2.4	5.8	+	+
19	3.7	7.2	3.8	6.6	+	+

IX. VIRULENCE AND ELECTROPHORETIC MOBILITY OF DISTILLED WATER SUSPENSIONS OF CORYNEBACTERIA

Theobald Smith described toxigenicity as the power of producing toxin *in vitro* and virulence as the behavior of diphtheria bacilli towards living tissue—a concept of virulence commonly held. Cary (1917) found that the virulence of a culture of diphtheria bacilli seemed to be of less importance in relation to penetration into tissues than the number of organisms injected. As will be observed from table 3 no apparent relationship exists between the electrophoretic mobility and recovery of organisms from several organs of the guinea pigs at postmortem as evidenced by observations that were not quantitative.

Many different experiments have been designed in the past to determine the relationship between the M.L.D. of toxin and the number of bacilli of a particular strain necessary to cause death of a guinea pig in four days. The results of Cary (1917) and of Barratt (1923) indicate no constant relationship between the M.L.D. and M.F.D. of the strains tested.

Before beginning specific experiments to determine parallelisms between the degree of toxigenicity and P.D. it was thought necessary to study the electrophoresis of washed diphtheria bacilli in distilled water and at the same time to determine the minimal

TABLE 3

P.D. and penetration

CULTURE	DOSE 1 CC.	DEATH	RECOVERY OF BACILLI FROM ORGANS					P.D. DISTILLED H_2O
			Heart's blood	Spleen	Liver	Adrenal	Kidney	
								μ/second
19	2 billion	24 hours	+	−	+	−	−	12.6
Park No. 8	2 billion	4 days	−	−	−	−	−	17.2
12	2 billion	2 days	−	−	−	−	−	10.6
26	2 billion	4 days	−	−	−	−	−	16.0
27	2 billion	2 days	−	+	−	−	−	17.0
49	2 billion	2 days	−	−	−	−	−	16.0
46	2 billion	4 days	−	−	−	−	−	17.0
15	2 billion	2 days	−	−	−	−	−	7.0

fatal dose for 250-gram guinea pigs of various strains of these bacilli suspended in the same menstruum.

Preparation of cultures

Twenty-four-hour growths of *C. diphtheriae* were washed from 2 per cent glycerol veal infusion agar with distilled water and centrifugated at high speed. The bacterial sediment was resuspended in distilled water and washed three times in all. With some strains it was necessary to break up the clumps by shaking thoroughly with glass beads.

The suspensions were standardized by the hemocytometer method of Cary (1917) and Barratt (1923). Although twenty-

four-hour cultures probably contain a maximal number of viable cells, the chief objection to microscopical methods of enumeration is that they fail to distinguish between living and dead bacilli. The suspensions in various dilutions were plated on glycerol agar as a check on the microscopical counts. Usually the plating method showed 25 per cent fewer organisms than did the counting chamber. An average of these two methods was taken as the final count for estimating the minimal fatal dose.

Objections to the use of distilled water as a menstruum in virulence experiments are overcome by the fact that no great loss of

TABLE 4

Electrophoretic mobilities of avirulent strains in broth and distilled water

CULTURE	P.D. 48-HOUR BROTH CULTURE	P.D. DISTILLED H_2O WASHED SUSPENSION	FERMENTATION (GLUCOSE)
	μ/second	*μ/second*	
10	9.4	30.0	−
2A	9.4	20.0	−
6	(12) (9.2)	21.0	−
44	8.5	28.0	−
34	6.2	24.0	−
39	6.2	26.0	−
13-1	5.7	26.0	−
32	5.9	30.0	+
41	4.5	34.0	+
33	4.4	34.0	+
14	5.6	25.0	+

Ten measurements of velocity (five with one and five with reversed orientation of the electrical field) were made at single level (497) (V_s) at room temperature. pH = 7.2.

viable bacilli was revealed by plate counts after distilled water suspensions had remained in the ice-box overnight. As a control, suspensions in 0.85 per cent NaCl solution held under the same conditions showed marked reductions in numbers.

Electrophoresis of viable cells in distilled water

Measurements of electrophoretic velocities of cells in forty-eight-hour broth cultures revealed sharp differences between non-fermentative, avirulent cultures and virulent, fermentative

strains. With the fermentative non-virulent strains, however, these mobilities were not so marked but were appreciably greater than those shown by virulent bacilli in the toxic broth. Measurements on these non-virulent strains in distilled water gave the results shown in table 4.

TABLE 5

Observed velocities of virulent strains in broth and distilled H_2O

CULTURE	P.D. 48-HOUR BROTH	P.D. (DISTILLED WATER)		
		First reading	Second reading	Third reading
	μ/second	μ/second	μ/second	μ/second
Park No. 8	(1.1)(0.85)(1.0)	12.1	7.2	3.7
19	1.9	7.5	2.6	12.0
23	2.8	3.6	4.0	4.0
50	2.8	11.7	5.1	5.9
47	2.9	9.0	6.6	7.0
31	3.1	3.4	5.6	5.6
49	3.1	10.0	6.0	15.0
26	3.2	3.4	6.0	6.0
48	3.2	10.9	8.0	8.0
28	3.4	6.8	8.5	8.0
36	3.4	11.3	11.3	11.3
27	3.4	11.3	7.5	7.5
20	3.4	17.0	5.6	5.65
5	3.5	8.0	6.3	6.3
B1	3.6	6.4	4.2	5.0
1	3.6	7.0	4.0	4.0
15	3.6	10.0	7.0	17.0
25	3.6	11.3	6.6	8.0
12	3.6	11.3	10.6	18.0
8	3.8	7.0	7.0	7.0
46	3.8	7.2	6.8	16.0
7	4.0	14.3	6.8	10.9
29	4.0	6.7	4.0	14.0
17	4.2	12.1	11.0	10.0
9	4.1	15.5	7.7	11.3

Mobility measurements on virulent bacilli washed three times and suspended in distilled water are given in table 5. It appears from table 5 that the electrophoretic mobilities of virulent bacilli are in every case of lower magnitude than those observed on avirulent corynebacteria. These strains had been cultured on

glycerol agar for about a year before these determinations were made. The apparently inconsistent results of the several readings are due in part to the addition of HCl or NaOH to adjust the suspensions to a pH of 7.0. Also, as has been pointed out by Winslow and Falk (1923), there is a tendency of bacteria to adjust the pH of their menstrua.

Virulence[2] *of distilled water suspensions of C. diphtheriae and electrophoretic mobility*

The standardized, washed suspensions were injected subcutaneously into 250- to 300-gram guinea pigs and the minimal fatal dose determined, i.e., the smallest number of bacilli causing death of the unprotected animal in four days. Mobility measurements were made on the suspensions as soon as possible after the inoculations were made. Nine dilutions of the suspension of bacilli were used. The total amount of each inoculation was 1 cc. of bacterial suspension.

A comparison of M.F.D. and P.D. is shown in table 6.

It is evident from the results shown in table 6 that there is no apparent relation between the number of organisms necessary to cause death in a 250-gram guinea pig and the mobility of cells of the same suspension of diphtheria bacilli in distilled water.

X. ELECTROPHORETIC MOBILITY AND TOXIGENICITY

It will be observed in table 1 above that the electrophoretic mobilities of broth cultures of the Park No. 8 strain are the lowest of any of the toxigenic strains of this collection. Owing to the high toxigenicity of this strain with the low mobilities observed on this and other virulent strains of *C. diphtheriae* it was assumed that the toxic substances elaborated in broth cultures might be responsible for the depression of mobility.[3] In table 2 it was

[2] Virulence here means ability to produce death in unprotected guinea pigs following subcutaneous injection of triply-washed diphtheria bacilli suspended in distilled water.

[3] It will be recognized that this does not follow from nor does it have any direct relation with the theoretical assumptions presented earlier in this paper. It is an independent assumption made to provide an *alternative* working hypothesis.

shown that the pH values of the menstrua do not influence the P.D. sufficiently to account for the differences in the observed velocities between toxigenic and non-toxigenic cultures. Two types of experiments were designed to test the validity of the

TABLE 6

A comparison of M.F.D. and P.D. (μ per second) of distilled water suspensions of bacilli

STRAIN	M.F.D.	P.D.	SOURCE
		μ/second	
19	500,000	12.0	Frank case
23	1,000,000	4.0	Suspected case
49	1,000,000	6.0	Suspected case
47	1,000,000	6.6	Suspected case
15	1,000,000	7.0	Frank case
27	1,000,000	7.5	Suspected case
31	5,000,000	5.6	Terminal
5	10,000,000	6.3	Suspected case
29	20,000,000	4.0	Suspected case
28	20,000,000	6.8	Suspected case
B1	50,000,000	4.2	Unknown
48	50,000,000	8.0	Suspected case
25	50,000,000	6.6	Terminal
36	50,000,000	11.3	Carrier
12	50,000,000	10.6	Carrier
50	50,000,000	(11.7) 5.9	Terminal
7	50,000,000	10.6	Terminal
9	50,000,000	9.8	Terminal
8	200,000,000	7.0	Carrier
17	200,000,000	11.0	Suspected case
1	500,000,000	4.0	Suspected case
26	2,000,000,000	6.0	Suspected case
46	2,000,000,000	7.2	Carrier
20	2,000,000,000	5.6	Suspected case
Park No. 8	2,000,000,000	17.0	Laboratory stock

inference: (1) To determine the M.L.D. of filtered, toxic broth and the P.D. of the cells in that menstruum on each of twelve consecutive days. (2) To determine the influence of salted-out, toxic material on the P.D. of a washed culture suspended in distilled water.

Experimental

Twelve 250 cc. Kolle flasks containing 50 cc. of 2 per cent Witte's peptone-veal infusion broth (pH 7.2) were inoculated with *C. diphtheriae* and the M.L.D. of the broth and the P.D. of the bacilli were determined on twelve consecutive days. The M.L.D. of the toxic broth was determined by using 250-gram guinea pigs and determining the smallest amount of toxin necessary to cause the death of the animal in four days after subcutaneous inoculation. Figures 1, 2 and 3. show the results of these determinations.

It will be observed in the figures that the mobilities of the bacilli fall as the culture ages and the toxin becomes more potent. However, the P.D. does not change much in value even though the primary toxigenicity has lessened, as will be seen in figure 2. In the light of experiments with heated toxin (fig. 5) this fairly permanent lowering of P.D. is to be expected, i.e., the substances excreted by *C. diphtheriae* may be non-toxic in part. The Park No. 8 strain seems to owe its regular toxigenicity to the fact that the toxin elaborated does not become less potent even up to twelve days. The other strains tested may have a high primary toxigenicity, but the toxin is unstable and deteriorates rapidly. Culture no. 19 very strikingly shows this effect (fig. 2).

The toxic material used to show the effect upon the P.D. of washed suspensions of bacilli in distilled water was obtained by saturating a seven-day filtered broth culture of Park no. 8 strain of *C. diphtheriae* with ammonium sulphate and skimming the salted-out toxin from the surface. This material was placed in a dialyzing membrane and dialyzed for twenty-four hours. The pasty material remaining was dried on filter paper and put in a dessicator over H_2SO_4. After drying, the toxin was ground in a mortar and stored in the ice-box. The M.L.D. of this material was determined for guinea pigs before use in the experiments indicated in figures 4 and 5. Dilutions of the toxin were made in distilled water containing triply-washed, virulent, diphtheria bacilli.

It is evident from figures 4 and 5 that salted-out toxic material from diphtheria cultures exerts a powerful depressing effect upon electrophoretic mobility. The heated toxoid material likewise

exerts a similar effect in lowering mobility. The material had been rendered non-toxic by heating in a water bath at 90°C. for forty-five minutes.

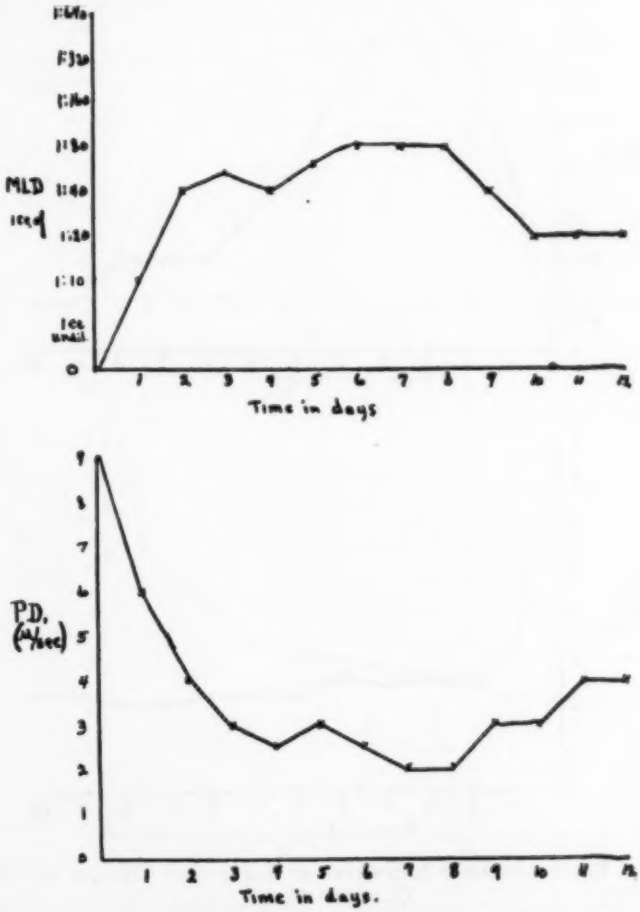

FIG. 1. ELECTROPHORETIC POTENTIAL AND TOXICITY OF CULTURE NO. 26, C. DIPHTHERIAE

Although it may be assumed that end products of bacterial metabolism exert some influence on the P.D. of the organism, the

characteristic difference in mobility between virulent and avirulent bacilli must be due to differences in the permeability of the

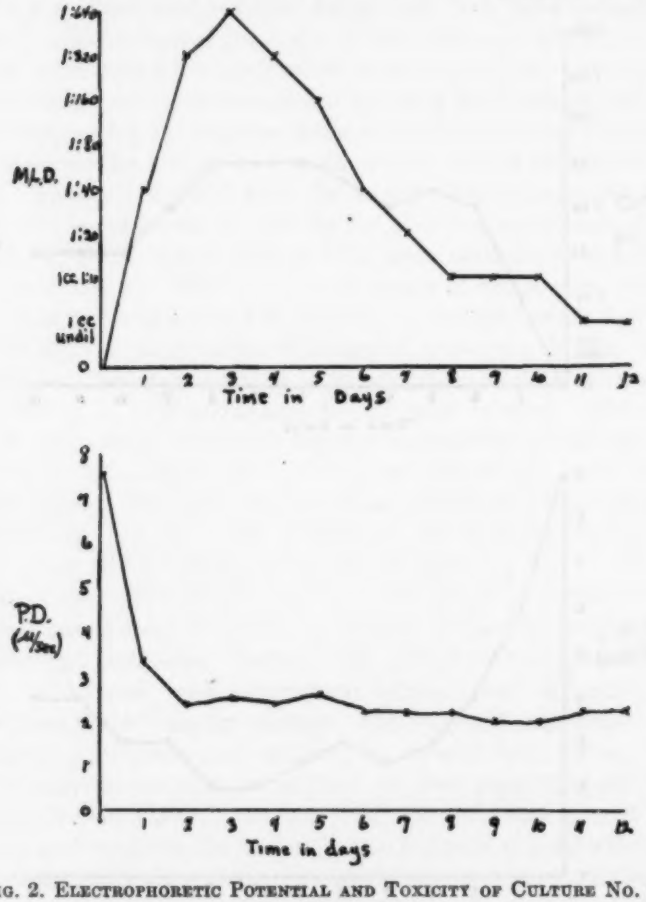

FIG. 2. ELECTROPHORETIC POTENTIAL AND TOXICITY OF CULTURE NO. 19, C. DIPHTHERIAE

bacterial cell itself.[4] It was shown that three-times-washed bacilli resuspended in distilled water also showed the two groups

[4] Assuming that the P.D. originates in membrane equilibria of the Donnan type as discussed on pages 373–5.

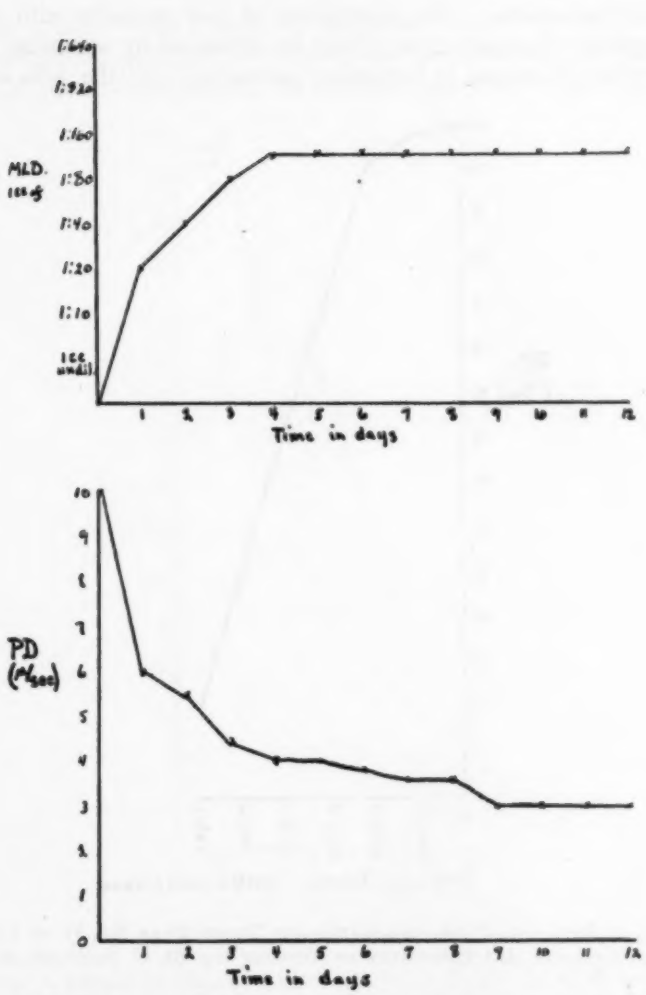

FIG. 3. ELECTROPHORETIC POTENTIAL AND TOXICITY* CULTURE PARK NO. 8, C. DIPHTHERIAE

* Broth buffered with 0.3 per cent K_2HPO_4 (pH 7.4)

of corynebacteria. The parallelism of low mobility with high toxigenicity in broth culture may be explained by assuming that with the differences in increasing permeability of the cells toxin

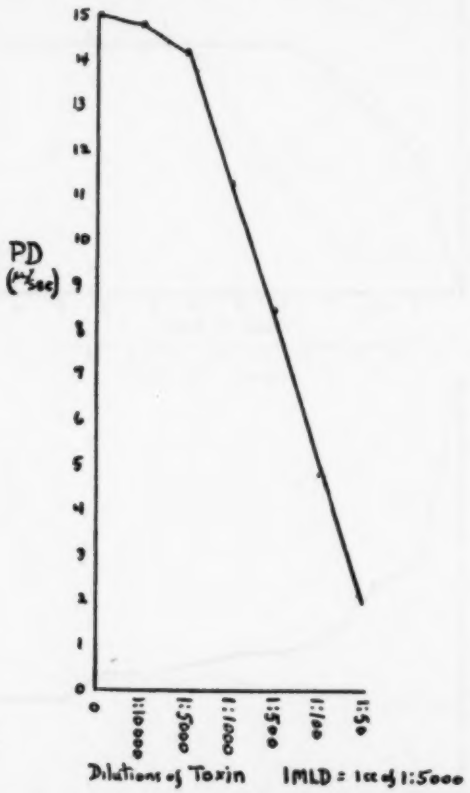

FIG. 4. EFFECT OF UNHEATED SALTED-OUT TOXIN (PARK NO. 8) ON P.D. OF DISTILLED H_2O SUSPENSION OF CULTURE NO. B1, C. DIPHTHERIAE

is more easily elaborated, and toxin forming materials more easily assimilated, and that high permeability is associated with low cataphoretic mobility. (See section V.)

Salted-out, uninoculated broth treated in the same way as described for toxic cultures also depresses potential in much the

same way as does heated or unheated toxin (fig. 6). Until toxin can be prepared in a purer state its true effect on the P.D. of the particles suspended in aqueous menstrua cannot be cer-

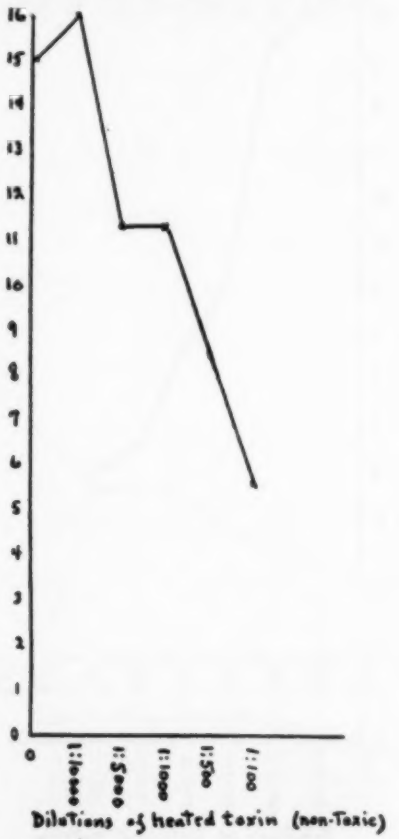

FIG. 5. EFFECT OF HEATED SALTED-OUT TOXIN (PARK NO. 8) ON P.D. OF DISTILLED WATER SUSPENSIONS OF CULTURE NO. B1, C. DIPHTHERIAE

tainly evaluated. Nor can the experimental findings be interpreted as validating the alternative, theoretical assumption stated on page 382.

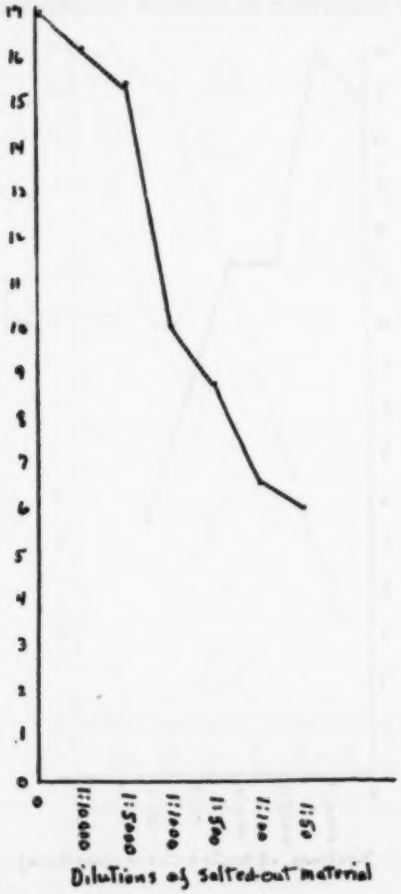

FIG. 6. EFFECT OF SALTED-OUT MATERIAL FROM UNINOCULATED BROTH ON P.D. OF DISTILLED H_2O SUSPENSION OF CULTURE NO. B1

XI. EFFECT OF CERTAIN SUBSTANCES ON TOXIN PRODUCTION AND ON ELECTROPHORETIC MOBILITIES

Inasmuch as high toxigenicity is a concomitant of low mobility of diphtheria bacilli in broth culture, it was thought necessary

FIG. 7. EFFECT OF $Ce(NO_3)_3$ ON P.D. OF WASHED SUSPENSION OF C. DIPHTHERIAE (STRAIN PARK NO. 8) SUSPENDED IN VEAL 2 PER CENT PEPTONE BROTH, pH 7.2

to determine the effect on toxigenicity of various reagents which had been shown in preliminary experiments to depress electrophoretic mobility.

Cerium nitrate, in the concentrations indicated in figure 7, was added to veal infusion 2 per cent peptone broth which was then inoculated with strain B1 of *C. diphtheriae*. The dilutions M/2500 to M/50,000 were used, but a potent toxin was produced

only in the cerium-free, control broth. The salt was toxic to the bacilli in M/50,000 concentration.

Botulinum toxins A and B increased the mobilities, with the result that toxin production in several flasks of veal peptone broth containing varying amounts of toxins, was delayed. Strong diphtheria toxin from the Park No. 8 strain could not be obtained in the presence of type A botulinum toxin. The guinea pigs used for testing toxigenicity of diphtheria toxin were first protected by injecting them with the homologous type of botulinum antitoxin. With washed suspensions of strain No. 47 suspended in beef heart broth (pH 7.2) the following mobilities were observed when botulinum toxins were added.

Botulinum toxins (pH 7.2), C. diphtheriae

	P.D.
	μ/second
100,000 M.L.D.'s Type A ($M7A_2$)	11.3
50,000 M.L.D.'s Type A ($M7A_2$)	8.0
Control beef broth (pH 7.2)	5.7
1,000 M.L.D.'s Type B (126.7)	6.2
Control beef broth	5.7

Cystine is often added to broth because it has been alleged that in the presence of 0.05 per cent of this substance toxin production by *C. diphtheriae* is enhanced. The experiment indicated in figure 8 was performed to determine the effect of cystine on broth suspensions of washed diphtheria bacilli. Twenty-four hour glycerol agar cultures were washed in distilled water by centrifugation and resuspended in veal infusion broth (pH 7.2) containing the amounts of cystine shown in figure 8. Although cystine is only slightly soluble at pH 7.2 (and is more soluble in more alkaline solutions) a definite depression of mobility was observed.

Forty-four newly isolated strains of *C. diphtheriae* were grown in veal, 2 per cent peptone broth to which cystine had been added in amounts of 0.5 gram to a liter. The broth was buffered with 0.3 per cent K_2HPO_4 (pH 7.4) and 50 cc. amounts were placed in 250 cc. flasks. After forty-eight hours' incubation at 37°C. the P.D. was determined for each strain. Mobilities ranging from

4.7 to 6.2 (μ per second) were quite often observed. These values are considerably higher than those observed in plain veal infusion or in peptone broth.

The effects of certain substances on toxigencity and P.D. of whole broth cultures and three-times-washed bacilli were studied with the hope that toxin production might be controlled by simply producing a favorable physico-chemical condition for the bacilli (a comparatively low P.D.) The following substances

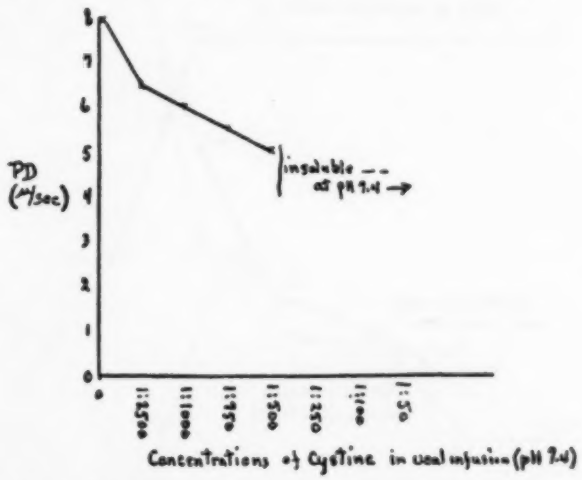

FIG. 8. EFFECT OF CYSTINE ON P.D. OF STRAIN NO. 27, C. DIPHTHERIAE SUSPENDED IN VEAL INFUSION BROTH

were added to 2 per cent Witte's peptone veal infusion broth (pH 7.2), in concentrations of 1.0, 0.1, 0.01, 0.001, 0.0001 and 0.00001 per cent:

Lanthanum nitrate	Calcium acetate
Vanadium sulphate	Sodium formate
Erbium nitrate	Sodium succinate
Thorium nitrate	Sodium oleate
"Didymium" nitrate[5]	Ammonium tartrate
Manganese chloride	Ammonium citrate
Calcium butyrate	Potassium valeriate

[5] A mixture of neodymium and praseodymium, free as possible from lanthanum.

The media containing these substances in the various amounts were added in 100 cc. amounts to 250 cc. flasks. They were each

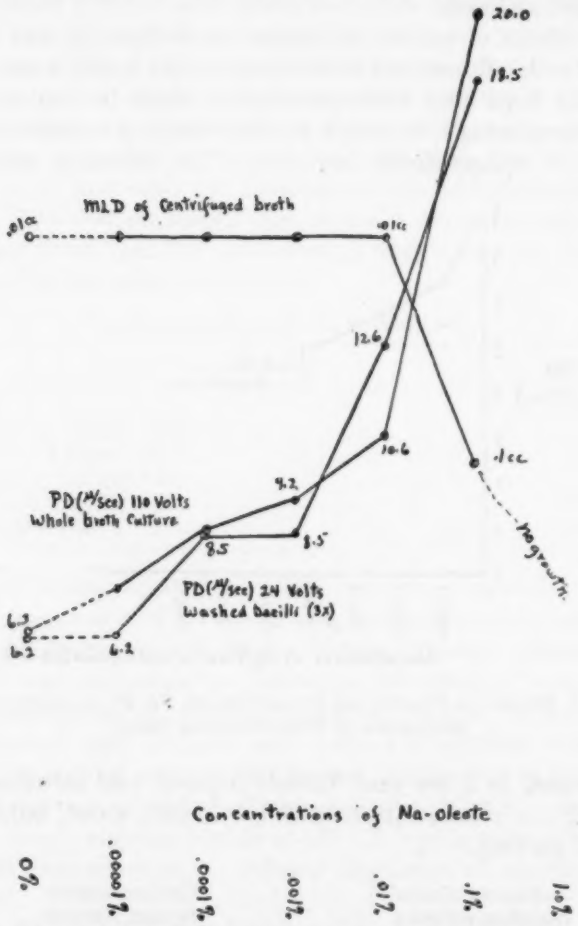

FIG. 9. EFFECT OF Na-OLEATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURES OF C. DIPHTHERIAE (PARK NO. 8)

inoculated with 1 drop of a one-day-old broth culture of Park No. 8 strain of *C. diphtheriae* and incubated at 37°C. for seven

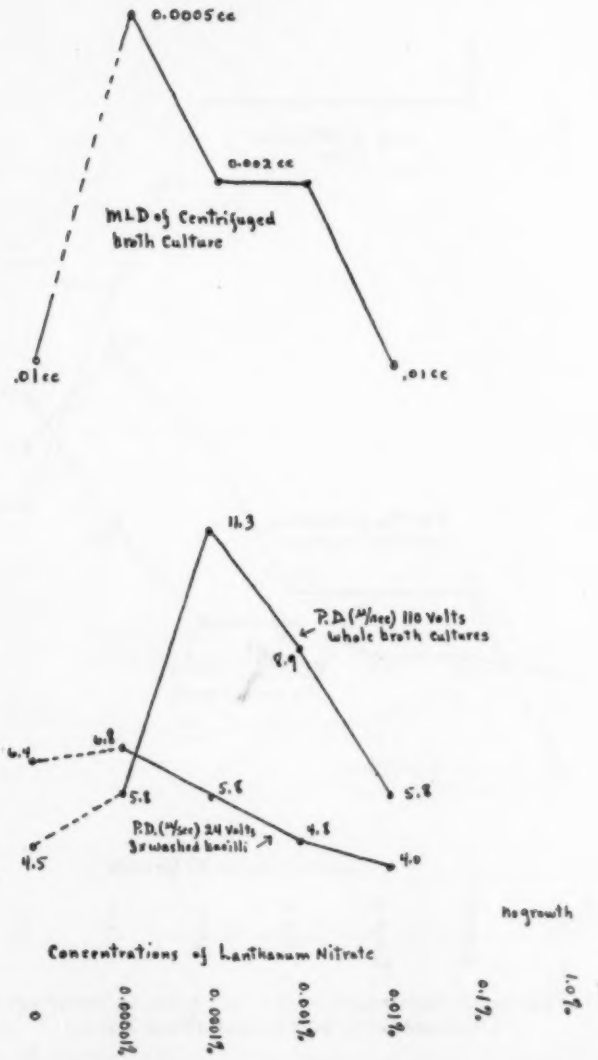

FIG. 10. EFFECT OF LANTHANUM NITRATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURES OF C. DIPHTHERIAE (PARK NO. 8)

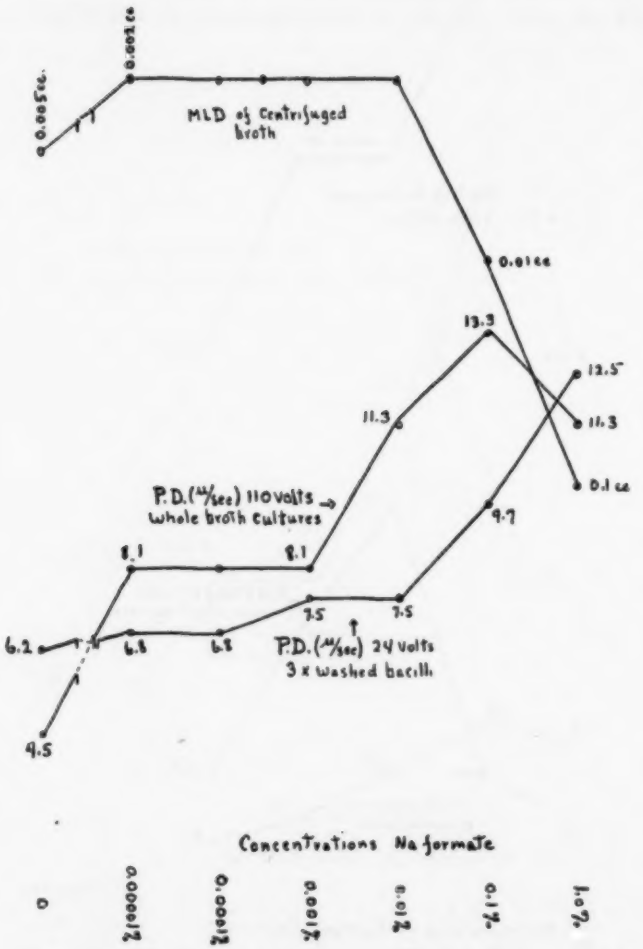

FIG. 11. EFFECT OF Na-FORMATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURES OF C. DIPHTHERIAE (PARK NO. 8)

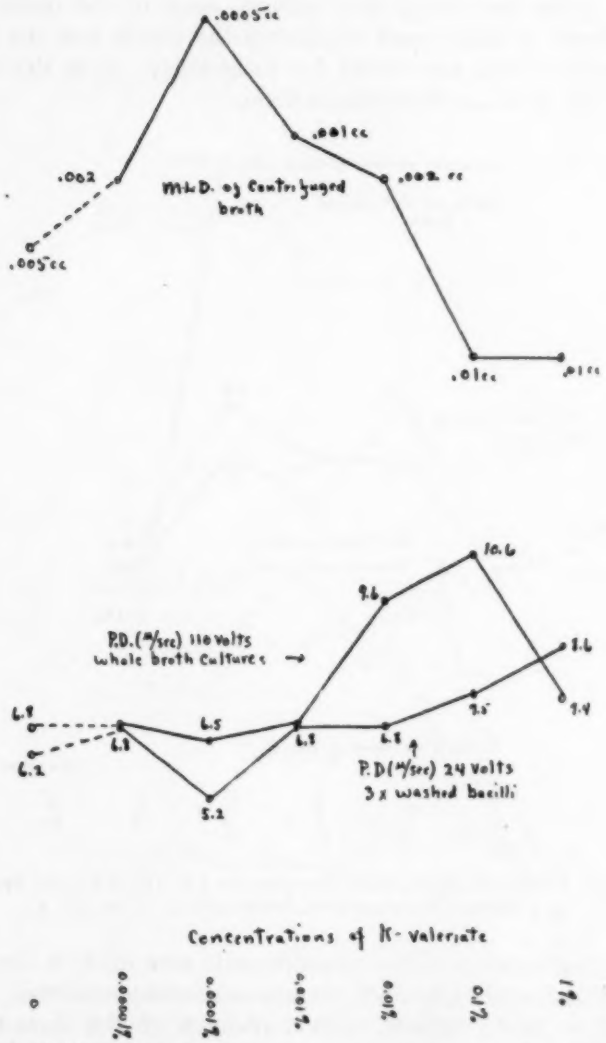

FIG. 12. EFFECT OF K-VALERIATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURES (PARK NO. 8)

days. After incubating each culture, some of the broth was centrifuged at high speed to remove the bacilli and the clear supernatant fluid was tested for toxigenicity, using the usual criteria for an M.L.D. of diphtheria toxin.

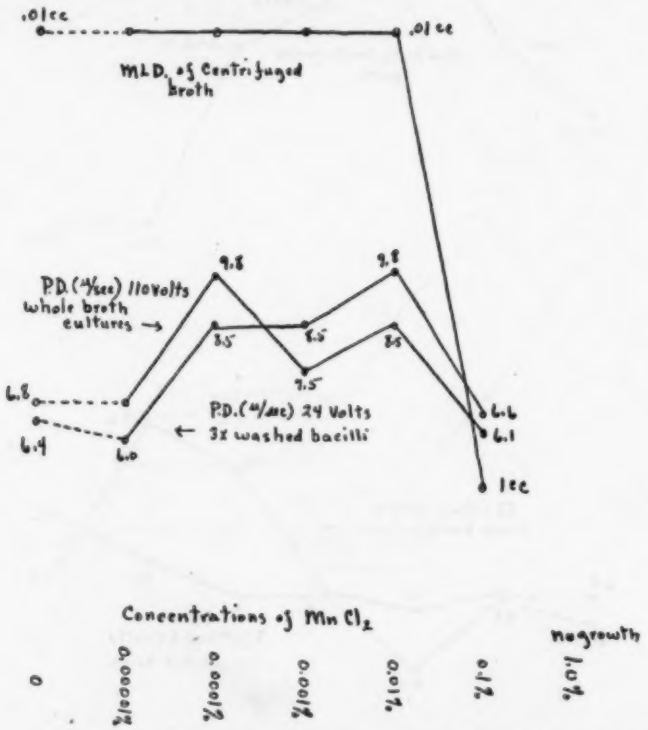

FIG. 13. EFFECT OF MANGANESE CHLORIDE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURES OF C. DIPHTHERIAE (PARK NO. 8)

Electrophoretic mobility measurements were made in the large apparatus described by Falk, Gussin and Jacobson (1925a), using the whole broth culture. Electrophoresis of the three-times-washed bacilli from these cultures was then observed in the simplified apparatus (described in the second following paper). Figures 9 to 22 show the results of these experiments. A maximal

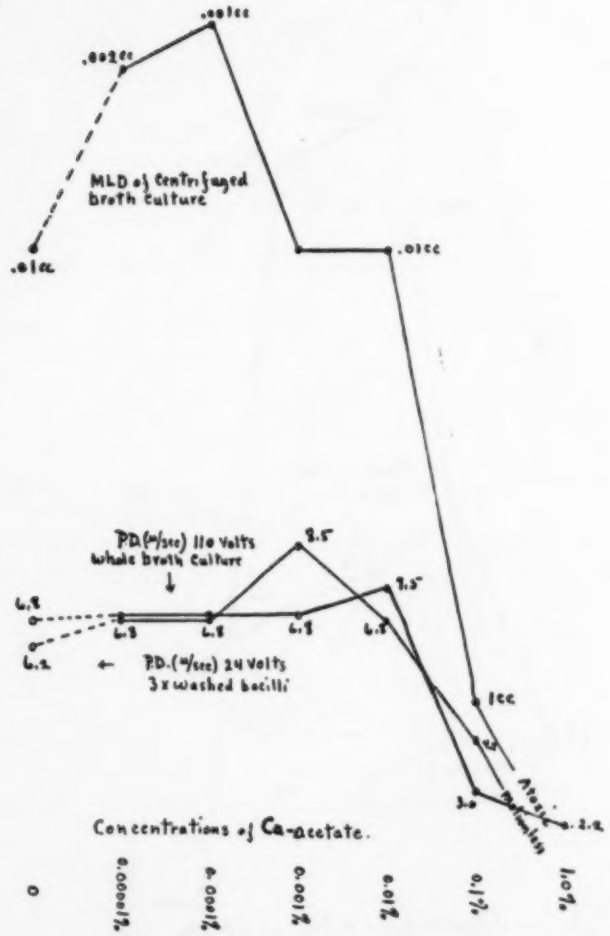

FIG. 14. EFFECT OF CALCIUM ACETATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURE OF C. DIPHTHERIAE (PARK NO. 8)

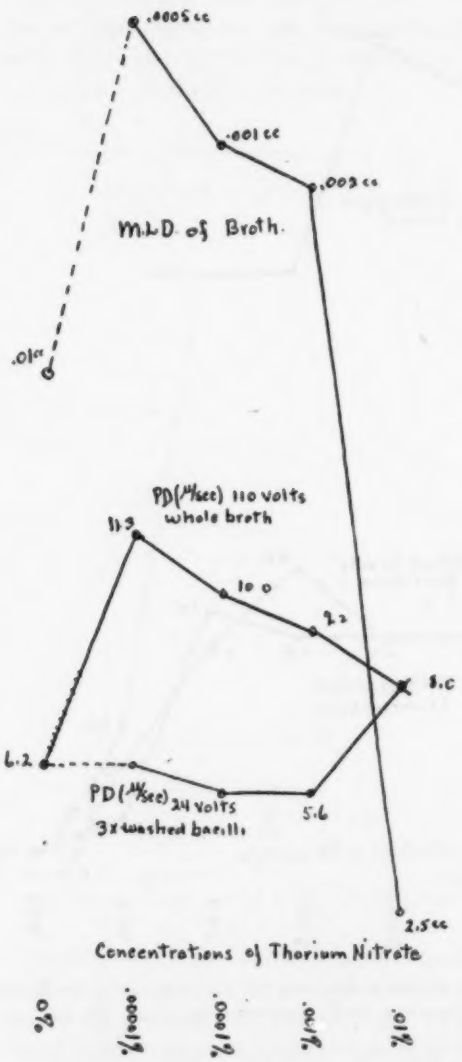

FIG. 15. EFFECT OF THORIUM NITRATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURES (PARK NO. 8)

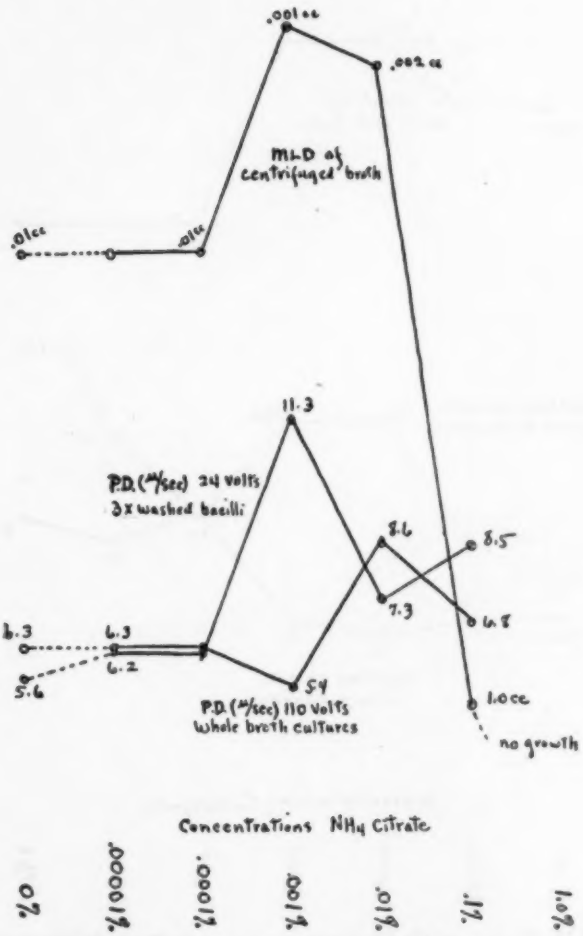

FIG. 16. EFFECT OF NH_4 CITRATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURES OF C. DIPHTHERIAE (PARK NO. 8)

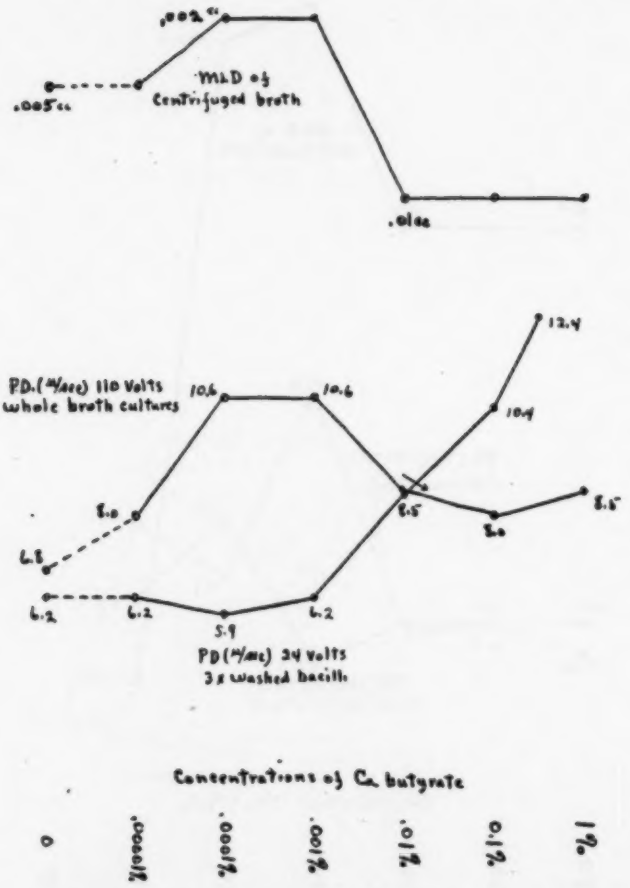

FIG. 17. EFFECT OF Ca BUTYRATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURES OF C. DIPHTHERIAE (PARK NO. 8)

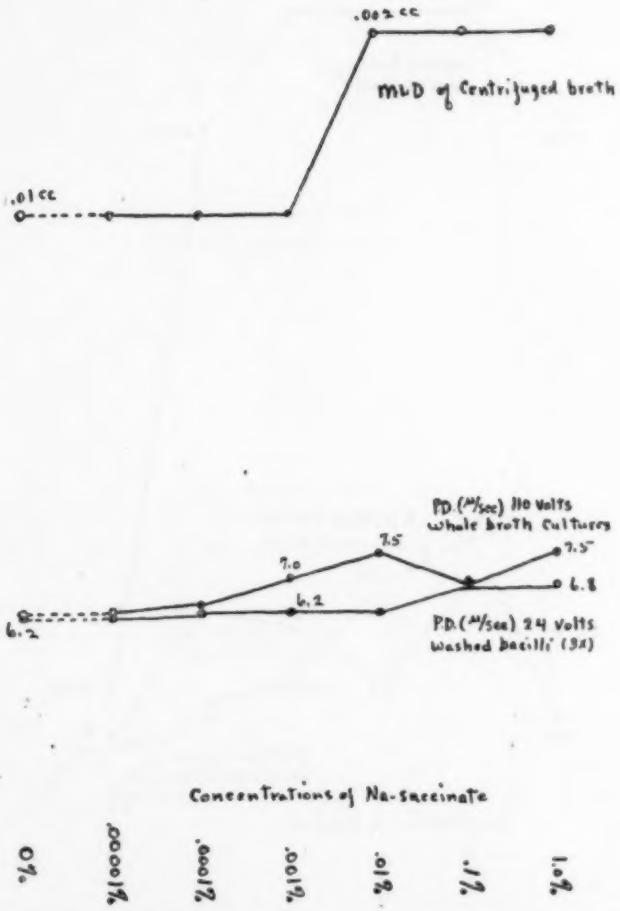

FIG. 18. EFFECT OF Na SUCCINATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURE (PARK NO. 8)

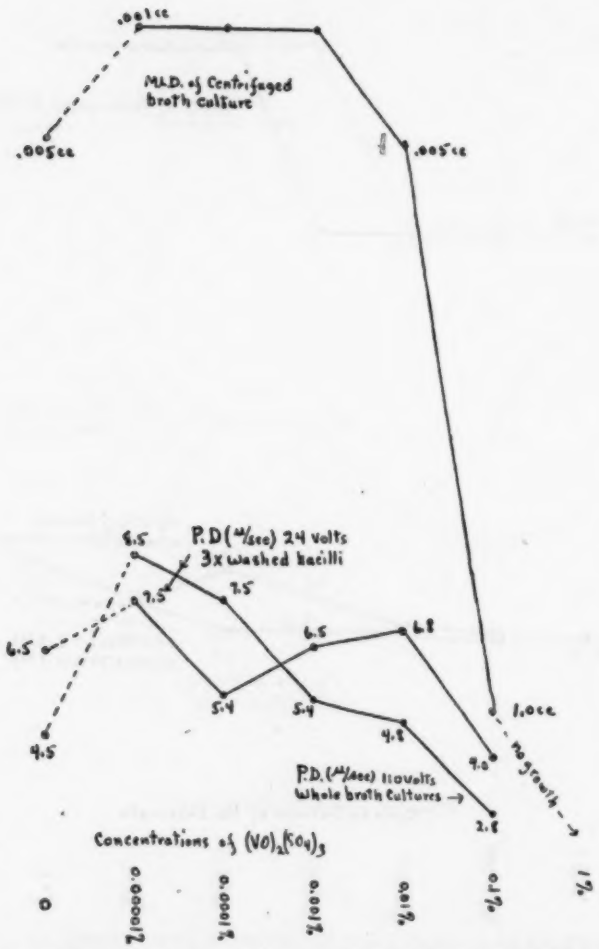

FIG. 19. EFFECT OF VANADIUM SULPHATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURES OF C. DIPHTHERIAE (PARK NO. 8)

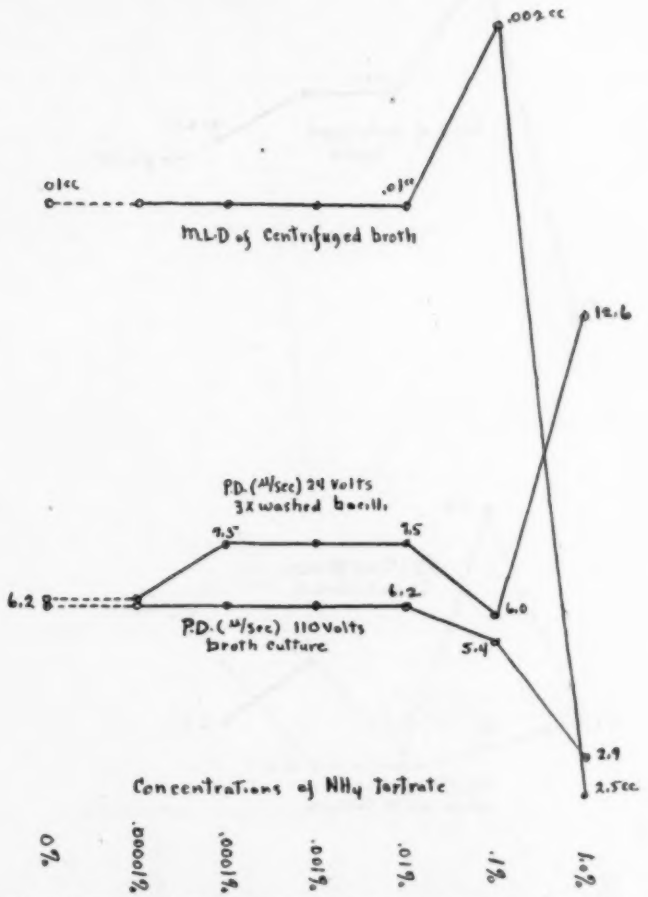

FIG. 20. EFFECT OF NH_4 TARTRATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURE (PARK NO. 8)

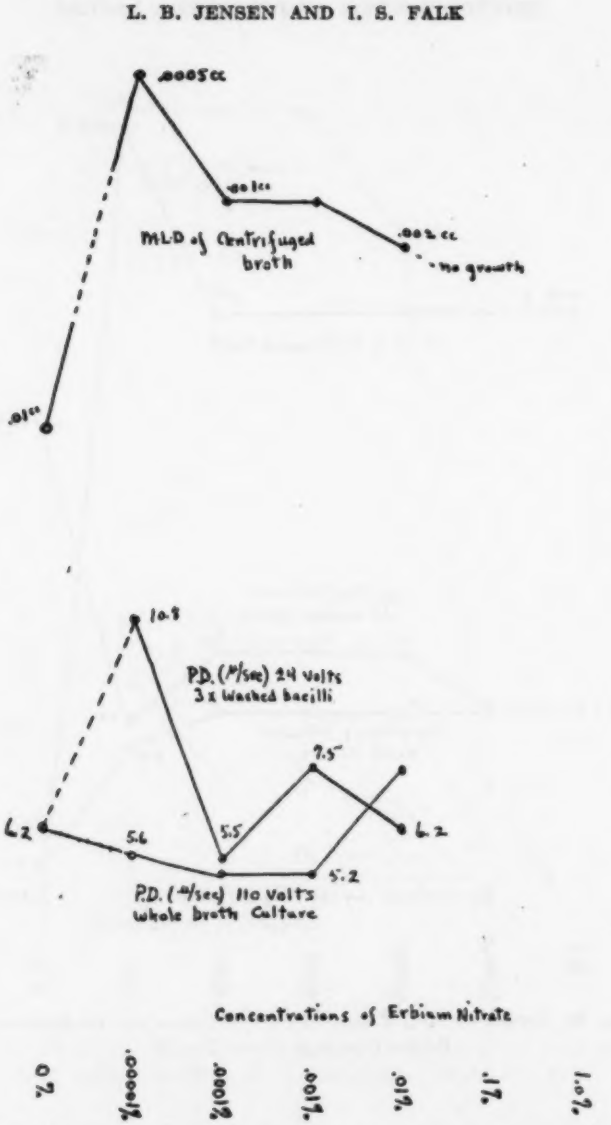

FIG. 21. EFFECT OF ERBIUM NITRATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURE OF C. DIPHTHERIAE (PARK NO. 8)

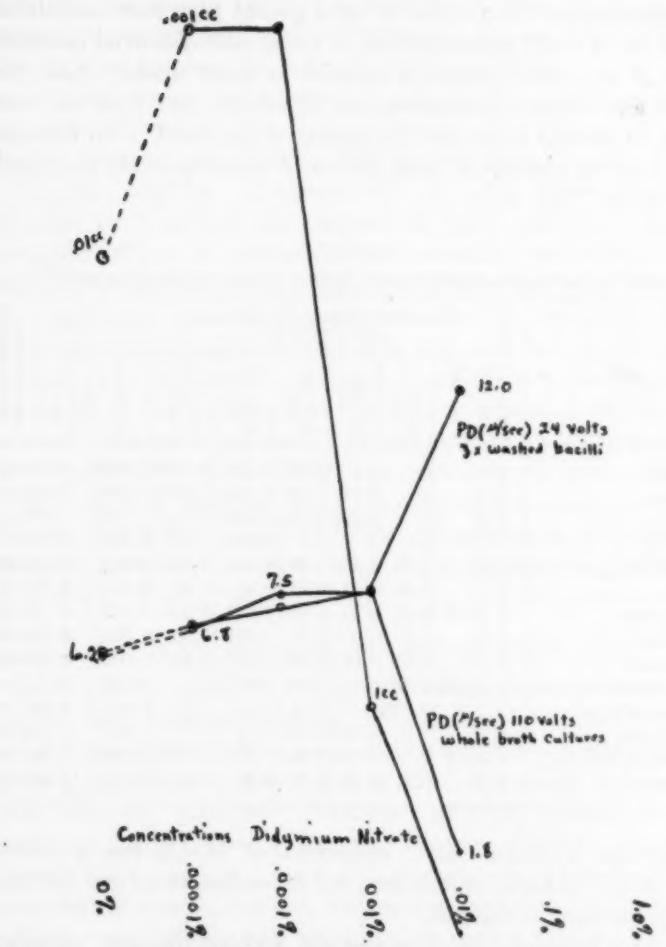

FIG. 22. EFFECT OF DIDYMIUM NITRATE ON P.D. AND M.L.D. OF SEVEN-DAY BROTH CULTURE (PARK NO. 8)

M.L.D. of 0.0005 cc.[6] was found in the lanthanum, erbium and thorium broths. A number of used guinea pigs were inoculated with 1 cc. of every concentration of these salts dissolved in broth. None of the salts killed the animals in three weeks. Also the lowest and highest concentrations of erbium and thorium were added to control toxin and the M.L.D. determined. No increase in toxicity was observed over that of a titration made four days previously.

TABLE 7

The effect of various concentrations of certain salts on toxigenicity and P.D.

SALT	VALENCE OF CATION	CONCENTRATIONS OF SALT AND M.L.D. PRODUCED IN EACH						MAXIMAL M.L.D.	P.D. AT MAXIMAL M.L.D.	CONTROL BROTH SALT FREE M.L.D.
		1 per cent	0.1 per cent	0.01 per cent	0.001 per cent	0.0001 per cent	0.00001 per cent			
$Th(NO_3)_4$	4	0	0	2.5	0.002	0.001	0.0005	0.0005	6.2	0.01
$La(NO_3)_3$	3	0	0	0.01	0.002	0.002	0.0005	0.0005	5.8	0.01
$Er(NO_3)_3$	3	0	0	0.002	0.001	0.001	0.0005	0.0005	5.6	0.01
$Di(NO_3)_3$	3	0	0	0	1.0	0.001	0.001	0.001	6.8	0.01
$(Va)_2(SO_4)_3$	3	0	1.0	0.005	0.001	0.001	0.001	0.001	5.4	0.005
$MnCl_2$	2	0	1.0	0.01	0.01	0.01	0.01	0.01	6.0	0.01
Ca-butyrate	2	0.01	0.01	0.01	0.002	0.002	0.005	0.002	5.9	0.005
Ca-acetate	2	0	1.0	0.01	0.01	0.001	0.002	0.001	6.8	0.001
Na-formate	1	0.1	0.01	0.002	0.002	0.002	0.002	0.002	6.8	0.005
Na-succinate	1	0.002	0.002	0.002	0.01	0.01	0.01	0.002	6.2	0.01
NH_4-tartrate	1	2.5	0.002	0.01	0.01	0.01	0.01	0.002	6.0	0.01
NH_4-citrate	1	0	1.0	0.002	0.001	0.01	0.01	0.002	5.4	0.01
K-valeriate	1	0.01	0.01	0.002	0.001	0.0005	0.002	0.0005	5.2	0.005
Na-oleate	1	0	0.1	0.01	0.01	0.01	0.01	0.01	12.6	0.01

Although Walbum (1921) believes that $MnCl_2$ has a stimulating effect on toxin production, our experiments do not indicate such properties for this salt.

Calcium butyrate, calcium acetate, sodium formate, ammonium tartrate, ammonium citrate, and sodium succinate do not produce marked increases in toxigenicity. Potassium valeriate,

[6] "If a toxin has a M.L.D. of 0.002 cc. it may be considered a strong toxin. A M.L.D. of 0.001 cc. is sometimes, though rarely, met with." "Diphtheria," Med. Res. Council, London, 1923, p. 124.

which is itself non-toxic to guinea pigs in 1 cc. amounts of 1:100 dilution, enhances toxin production. Sodium oleate which reduces surface tension and increases P.D., in accordance with our theory, does not permit a potent toxin to be formed.

Table 7 shows the effects of various concentrations of the reagents studied on the toxigenicity of the Park no. 8 strain. It will be noted in table 7 and in figures 9 to 22 that striking, although irregular enhancements of toxin production were effected by many of the reagents studied, and that similarly irregular effects on electrophoretic mobility were recorded. It must be recalled, however, that the cultures had been incubated for seven days before they were tested for toxicity or for electrophoretic mobility. By the growth and activity of the microorganisms, many complex changes of unknown nature have been produced in the media and it is not surprising that the effects of the test substances on mobility, measured seven days after they have operated physiologically, are confused. The simple, clear effects of such reagents upon P.D. are well known (cf. fig. 7). The striking thing is that by the addition of some of them broth cultures have been prepared with almost unprecendented toxicities (M.L.D. = 0.0005 cc.).

XII. SUMMARY AND CONCLUSIONS

1. Forty-five virulent and five avirulent strains of diphtheria bacilli were indistinguishable when tested upon the following substances; fucose, d-arabinose, a-mannoheptose, a-glucoheptose, cellose, melibiose, trehalose, melezitose, rhamnose, xylose, levulose, glucose, galactose, mannose, sucrose, lactose, maltose, raffinose, inulin, starch, glycogen, dextrin, dulcitol, erythritol, d-mannitol, adonitol, d-arabitol, l-arabitol, glycerol, arbutin, a-methyl glucoside, salicin, amygdalin, mannonic acid lactone, gulonic acid lactone and glucosamine hydrochloride. Of these test substances glucose, levulose, galactose, maltose, dextrin and glycerol were fermented alike by the 50 strains. Acid was not produced from any of the other test substances except glucosamine-HCl. Five of the virulent and one of the avirulent cultures failed to ferment this substance.

2. From certain theoretical assumptions it was anticipated that electrophoretic mobilities would be different for strains of diphtheria bacilli of different degrees of virulence.

Measurements of electrophoretic potentials on forty-eight-hour veal broth cultures of toxigenic strains gave potential differences of 4.2 to 1.0 μ per second with an average of 3.4 μ per second.

Measurements of electrophoretic potentials on forty-eight-hour veal broth cultures of avirulent strains which ferment sucrose gave higher values (P.D. 12.1 μ per second). Non-fermenting avirulent cultures (*C. hoffmanni*) gave potential differences of 7.9 to 10.0 μ per second. Strains with P.D. of intermediate values (4.4 to 6.2 μ per second) were according to morphological and biochemical characteristics typical diphtheria bacilli but were not virulent or toxigenic.

3. Inasmuch as P.D. is known to be a function of pH, the indicated experiments were performed and it was found that the characteristic potential differences found on virulent and avirulent bacilli were not due to this factor.

4. There is apparently no relationship between the electrophoretic potential and recovery of organisms from several organs of guinea pigs *post mortem* as evidenced by observations that were not quantitative.

5. Distilled water suspensions of corynebacteria washed three times in distilled water gave the following potentials: all avirulent strains P.D. = 20.0 to 34.0 μ per second-110 volts; all virulent strains range in mobilities between 2.6 and 11.3 μ per second.

6. Apparently no parallelism exists between the minimal fatal dose of washed bacilli suspended in distilled water and their P.D. in the same menstruum.

7. The lowest P.D. values observed in broth cultures were found on the Park no. 8 strain of *C. diphtheriae* which has the greatest toxin producing powers of any strain used in our studies.

8. The degree of toxigenicity parallels P.D., i.e., low potential is a concomitant of high toxigenicity.

9. Salted-out, purified, toxic materials depress P.D. in a quantitative manner but this finding is of little significance until "toxin" can be obtained in a purer state.

10. The power of virulent strains to produce toxin diminishes with increases in P.D. and is enhanced when the P.D. is decreased with certain reagents.

11. If P.D. and M.L.D. of broth cultures are determined daily for twelve days, an inverse parallelism is shown to exist for certain cultures and not for others.

12. Throughout the studies reported here (and in the following papers) it was found that toxigenicity appears to vary in a quantitative (and not in a qualitative) manner among diphtheria bacilli.

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ELECTROPHORESIS OF DIPHTHERIA BACILLI

II. MICRO-ELECTROPHORESIS AND THE DIFFERENTIATION OF VIRULENT AND NON-VIRULENT DIPHTHERIA BACILLI

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The experiments thus far reported[1] have shown that there exist roughly parallel differences in P.D. between the toxigenic and non-toxigenic corynebacteria. The electrophoretic mobility varies inversely with toxigenicity, i.e., low potential is a concomitant of high toxigenicity. The studies reported here were designed to attempt an application of electrophoretic measurements to the determination of "virulence" on cultures submitted to the Bureau of Laboratories and Research in the Chicago Department of Health. It was our intent first to determine how closely "virulence" determinations made by electrophoretic measurements would conform to guinea pig tests, and, second, if the results were satisfactory to attempt the development of an electrophoretic technique that would be simple and rapid as well as reliable.

Before undertaking these routine tests it was necessary to find a broth medium which would permit maximum toxin production in the shortest time and which would not contain substances that would interfere with or cause equivocal mobility readings. Cystine was tried with the results that have been described in the preceding paper. The following media were also tried: veal infusion + 2 per cent Merck's peptone without NaCl; plain veal infusion; veal infusion + Fairchild peptone without salt; veal

[1] Cf. the preceding paper, in this JOURNAL, by Jensen and Falk.

TABLE 1

Electrophoretic mobilities: μ per second

CULTURE	GLASS (48-HOUR PEPTONE MERCK)	QUARTZ (48-HOUR PEPTONE MERCK)	QUARTZ (24-HOUR VEAL)	GLASS (48-HOUR VEAL)	GLASS (48-HOUR PEPTONE BUFFERED)	GLASS (48-HOUR PEPTONE CYSTINE BUFFERED)	VIRULENCE (BY GUINEA PIG TEST)
1	5.1	13.0	8.5	6.2	6.8		+
2	4.0	10.0		4.0	4.5	5.6	+
3	4.5	8.0		3.4	4.0	4.0	+
4	5.6	9.0	8.0	3.4		4.2	+
5				5.6	4.5	4.2	+
6	4.8	7.5		7.5	lost		+
7				6.6	8.5	4.8	+
8	3.0	9.0		2.8	4.4		+
9				4.8	3.7	4.0	+
10	4.2	6.8	7.7	2.1	3.4	4.0	+
11				3.7		4.2	−
12	3.4	10.0	7.4	4.8	4.2	6.1	−
13	6.8	11.3	9.0	7.5		6.1	+
14	4.5	8.5	7.4	3.6	4.2	4.7	+
15	5.2	9.0	10.0	6.2	6.8	4.2	+
16	4.0	6.8		3.4	4.0	4.7	+
17	3.9	8.0	7.0	7.5		4.7	+
18				3.4		4.1	+
19	5.2	8.5	7.9	3.5		5.1	+
20	4.9	6.2	6.4	6.4		5.2	+
21				4.8	4.5	6.1	+
22	3.4	10.0	10.0	10.0	4.5	5.2	+
23	4.2	8.5		6.8	3.4	3.4	+
24	6.2	7.7		3.4		4.2	+
25					6.8	6.8	−
26					4.2	6.6	+
27					4.4	4.4	+
28					4.2	4.2	+
29					3.7	3.7	+
30					3.7	2.8	+
31					4.2	3.7	+
32						4.4	+
33						5.6	+
Park No. 8	3.0	8.0	7.4	2.4	5.5	6.5	+

infusion + 2 per cent Difco peptone buffered with Na_2HPO_4; the same medium with cystine; veal infusion + 2 per cent Merck's peptone with cystine and veal infusion + 2 per cent Witte's peptone without salt. All the media were adjusted to pH 7.4.

Of these media, the veal 2 per cent Witte's peptone gave fairly constant results and was used in the final determinations.

Table 1 shows values obtained in casting about for a suitable medium to be used as a standard for this technique. Only a

TABLE 2

Electrophoretic mobilities in distilled water suspensions (24 hour glycerol agar cultures)

CULTURE	QUARTS CELL P.D.	GLASS CELL	VIRULENCE
	μ per second		
1	17.0	28.0	+ (mixed)
2	17.0	17.0	+
3	17.0	19.0	+
4	17.0	19.0	+
5	34.0	22.6	+
6	15.0	28.0	+
7	34.0	28.0	+ (mixed)
8	17.0	17.0	+
9	17.0	28.0	+
10	17.0	17.0	+
11	17.0	28.0	+
12	18.0	22.6	+
13	17.0	22.6	+
14	17.0	17.0	+
15	16.0	22.6	+
16	13.0	17.0	+
17	34.0	34.0	+
18	17.0	22.6	+
19	17.0	17.0	+
20	20.0		+
21	17.0	17.0	+
22	18.0		+
23	17.0	19.0	+
24	16.0	17.0	+
25		24.0	−
26		20.0	+
27		18.8	+
28			+
29		17.0	+
30		17.7	+

few of the tests are listed. Determinations made with a quartz cataphoresis cell were also made along with the tests in a glass apparatus. The measurements in the quartz apparatus did not

TABLE 3

Identification of unknown corynebacteria

CULTURE	P.D. (48-HOUR 2 PER CENT WITTE'S PEPTONE BROTH)	VIRULENCE BY ELECTROPHORESIS	VIRULENCE ACCORDING TO GUINEA PIG TESTS
	μ per second		
1	4.8	+	+
2	4.8	+	+
3	4.8	+	+
4	4.2	+	+
5	4.0	+	+
6	4.0	+	+
7	4.5	+	+
8	4.0	+	+
9	4.0	+	+
10	4.0	+	+
11	5.2	−	−
12	5.7	−	−
13	4.8	+	+
14	4.5	+	+
15	4.8	+	+
16	4.5	+	+
17	4.7	+	+
18	6.0	+	+
19	4.5	+	+
20	4.7	+	+
21	4.8	+	+
22	4.8	+	+
23	4.0	+	+
24	4.0	+	+
25	11.3	−	−
26	4.8	+	+
27	4.4	+	+
28	4.2	+	+
29	4.0	+	+
30	4.0	+	+
31	3.7	+	+
32	4.5	+	+
33	4.8	+	+
34	4.0	+	+
35	11.3	−	−
36	11.3	−	−
37	4.2	+	+
38	8.0	−	−
39	4.0	+	+
40	4.0	+	+
41	4.0	+	+

TABLE 3—*Continued*

CULTURE	P.D. (48-HOUR 2 PER CENT WITTE'S PEPTONE BROTH)	VIRULENCE BY ELECTROPHORESIS	VIRULENCE ACCORDING TO GUINEA PIG TESTS
	μ per second		
42	4.0	+	+
43	6.8	−	−
44	4.0	+	+
45	4.2	+	+
46	4.8	+	+
47	4.2	+	+
48	4.8	+	−
49	4.2	+	−
50	4.8	+	+
51	3.7	+	+
52	4.5	+	+
53	3.4	+	+
54	4.8	+	+
55	3.7	+	+
56	4.8	+	+
57	4.8	+	−
58	4.8	+	+
59	4.2	+	+
60	3.4	+	+
61	4.8	+	+
62	3.4	+	+
63	3.4	+	+
64	4.5	+	+
65	4.2	+	+
66	4.4	+	+
67	4.4	+	+
68	4.4	+	+
69	4.2	+	+
70	4.4	+	+
71	11.3	−	−
72	5.0	+	+
73	4.0	+	+
74	4.8	+	+
75	4.8	+	+
76	4.8	+	+
77	3.2	+	+
78	4.8	+	+
79	4.4	+	+
80	4.4	+	+
81	4.4	+	+
82	4.8	+	+
83	4.0	+	+

TABLE 3—*Continued*

CULTURE	P.D. (48-HOUR 2 PER CENT WITTE'S PEPTONE BROTH)	VIRULENCE BY ELECTROPHORESIS	VIRULENCE ACCORDING TO GUINEA PIG TESTS
	μ per second		
84	No culture		
85	4.0	+	+
86	4.8	+	+
91	6.8	−	−
92	4.8	+	+
93	4.8	+	−
94	4.8	+	+
95	3.8	+	+
96	3.8	+	+
97	4.2	+	+
98	4.0	+	+
99	4.2	+	+
100			
101	4.0	+	+
102	4.0	+	+
103	6.0	−	−
104	4.4	+	+
105	11.3	−	−
106	3.8	+	+
107	3.2	+	+
108	3.8	+	+
109	4.2	+	+
110	11.3	−	−
111	6.8	−	−

parallel those made in the glass cell and the use of the quartz apparatus was discontinued.

In table 2 are listed the measurements obtained with distilled water suspensions of eighteen-hour glycerol agar (0.5 per cent beef serum) slants. For reasons which we have not discovered, freshly isolated bacilli have higher mobilities in distilled water than have strains which have been cultivated in the laboratory for six months or more.

In this work it was found that pseudo-diphtheria bacilli were often introduced in the transfers of several colonies from Loeffler's medium. These mixed cultures in distilled water gave equivocal mobilities. When each colony is transferred to a separate single tube, these results have not been noted except in a few instances in the present series.

Using the large apparatus, mentioned in the preceding paper, for electrophoresis determinations, P.D. (μ per second) 5.0 was taken as the criterion for differentiating toxigenic from non-toxigenic bacilli.[2] All forty-eight-hour broth cultures with a P.D. (μ per second) higher than 5.0 were considered non-toxigenic; those lower than P.D. (μ per second) 5.0 were called toxigenic.

In table 3 are listed the results of potential measurements with the diagnoses from them, and the results of virulence tests conducted by the guinea pig method, injecting subcutaneously 0.5 to 2.0 cc. of a salt solution suspension from the original Loeffler's serum culture. The results of each test were unknown to the individuals performing the other until after the completion of all of the cataphoresis experiments.

In this series of 105 unknown cultures of corynebacteria the following findings were obtained: *a*, 88 were *virulent* by both electrophoresis and by guinea pig tests; *b*, 13 were *non-virulent* by both tests; *c*, 4 were *virulent* by electrophoresis and *non-virulent* by guinea pig tests.

An electrophoretic mobility of 5.0 μ per second had been taken as the dividing line between the strains that were to be called "virulent" and those that were to be called "non-virulent." It is significant to note that of the four cultures on which the electrophoretic and the guinea pig tests disagreed, three had given mobilities of 4.8 μ per second (and were just barely in the "virulent" category by this test), and the fourth had given a mobility of 4.2 μ per second. There were no reports available on guinea pig retests. Thus, we may say that the electrophoretic mobility method erred only four times in 105 and in each case on the side of sanitary safety by calling a strain "virulent" which by a guinea pig test was "non-virulent."

Many of these cultures had been taken from cases of scarlet fever and ear infections and, as the mobilities show, are all low grade toxin producers.

[2] We have already called attention (in the preceding paper) to the objections against the assumption of a discontinuity in virulence. Yet such an assumption must be made if the diagnostic laboratory wishes to say that a culture is either "virulent" or "non-virulent."

ELECTROPHORESIS OF DIPHTHERIA BACILLI

III. THE DEVELOPMENT OF A SIMPLIFIED TECHNIQUE FOR ELECTROPHORETIC MEASUREMENT OF THE VIRULENCE OF DIPHTHERIA BACILLI

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Because of the somewhat complex form of the apparatus used in the electrophoresis measurements reported in the two preceding papers of this series, and the time and number of operations involved in growing and preparing cultures for the test, we undertook the task of attempting to develop reliable, simplified methods that might be utilizable in the public health laboratory.

Several types of depression slide cells have been devised. The first attempt with point platinum electrodes did not provide a suitable method because of polarization and electrolysis at the electrodes when broth media were used as suspending menstrua. Even though platinum black was deposited upon the electrodes, gas bubbles soon appeared and caused equivocal mobilities.

Another type of cell with non-polarizing electrodes was devised resembling the cell described by Taylor.[1] Although it is not practical for routine laboratory tests, uses for it may be found elsewhere.

The type of cell finally adopted is made as follows: Glass slides are cut into desired sizes and cemented to a thick glass slide forming a rectangular cell with grooves providing places for platinum electrodes. In figures 1 and 2 are shown the top and side views of the cell with dimensions and arrangement of apparatus.

[1] Taylor, C. V., Proc. Soc. Exper. Biol. and Med., 1925, **23**, 147. Cf. also Gelfan, S., ibid., 1926, **23**, 308.

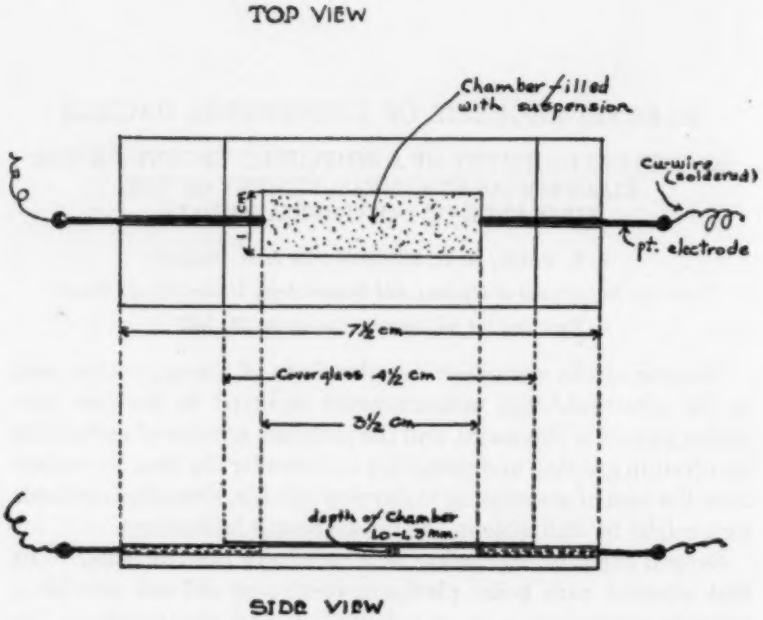

FIG. 1

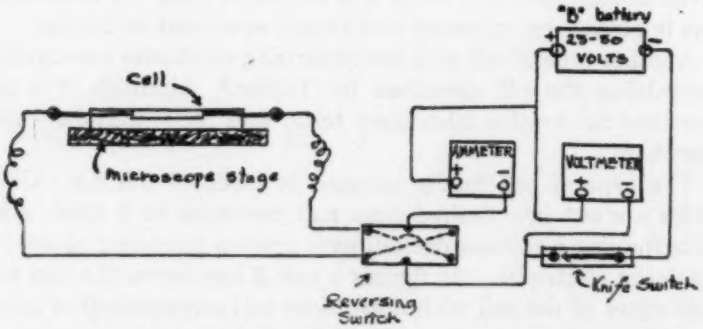

FIG. 2. WIRING DIAGRAM FOR THE SIMPLIFIED ELECTROPHORESIS CELL

FIG. 3. IMPROVED FORM OF THE "SLIDE CELL" FOR SUSPENSIONS IN DISTILLED WATER

The platinum electrodes are cemented part way in the grooves with deKhotinsky cement, leaving 0.5 cm. or less of the end of the wire uncovered.

The cell is cleaned and filled with distilled water suspensions of bacteria. A large coverglass is pressed tightly[2] over the cell and excess water is removed by blotting. The whole slide is now mounted on a microscope provided with a compensating ocular (25×) with a Sedgwick–Rafter micrometer (each large square being 34 μ square on the observed field) and an 8 mm. objective. The electrodes are connected with a source of direct current. (We use a 45 volt dry cell battery for routine work.) In figure 3 we show the simple slide cell in a form improved by Dr. Klopsteg of the Central Scientific Company of Chicago.

PROOF OF TRUE ELECTROPHORESIS

Preliminary tests on this simple cell showed the usual differences in velocity between virulent and avirulent diphtheria bacilli observed in previous tests with the large apparatus. It was necessary to prove that these preliminary results were not a matter of chance and that the simplified cell showed true electrophoresis. This was tested by observing whether the velocity was proportional to the impressed voltage. Using a three-times-washed forty-eight-hour broth culture of *C. hoffmanni* with the source of potential at 114 volts, the following results were obtained. The culture (in water) was an old one that had been held in the ice chest several days (table 1).

It is apparent from this experiment and others that follow that the cell shows true cataphoresis as tested by this method.

After placing the cell in water overnight, washing in 50 per cent HCl and rinsing in distilled water, a similar test was conducted, but the forty-eight hour broth culture of *C. hoffmani* was used after one washing with water. As will be seen in table 2 remarkably true electrophoresis was again obtained.

[2] It was found that observed velocities varied greatly unless the coverglass was fitted tightly on the slide. This effect is due to the resulting change in the position of the V_s (stationary layer) from the calculated one, if the depth of the cell is increased or decreased.

TABLE 1

RESISTANCE	VOLTS	MILLIAMPERES	MOBILITY	MOBILITY
ohms			*μ/second*	*μ/second/volt*
0	114	8.0	77.3	0.678
6,200	80	5.8	49.3	0.616
18,900	50	3.5	32.4	0.648
42,400	30	2.0	20.2	0.674
Average				0.667

Ten measurements of velocity (5 with one and 5 with reversed orientation of the electrical field) were made at V_s 1262.

TABLE 2

IMPRESSED VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
30	12.1	0.247
50	18.9	0.264
80	29.5	0.271
100	37.8	0.264
114	44.7	0.255
Average		0.260

TABLE 3

Culture no. 111

VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
114	48.2	0.422
100	40.0	0.400
80	29.9	0.374
60	22.2	0.370
50	17.1	0.342
40	13.6	0.340
30	9.0	0.300
20	6.23	0.312
13	4.8	0.369
Average		0.359

That these experiments were conducted with cultures that had been washed only once should be especially noted.

TABLE 4

Culture no. 85

VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
114	72.2	0.633
100	64.7	0.647
80	54.7	0.683
60	38.2	0.637
40	26.3	0.665
30	20.4	0.680
20	12.9	0.644
13	8.35	0.643
Average		0.654

TABLE 5

Culture no. 86

VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
100	82.4	0.824
80	63.8	0.798
50	37.0	0.739
40	27.9	0.697
30	24.4	0.813
20	11.7	0.585
13	7.9	0.607
Average		0.723

TABLE 6

Culture no. 110

VOLTAGE	MOBILITY	MOBILITY
	μ/second	*μ/second/volt*
114	66.3	0.582
100	64.2	0.642
80	48.6	0.608
50	28.7	0.574
30	17.0	0.567
13.5	7.9	0.585
Average		0.593

Once washed forty-eight-hour broth cultures of Nos. 111, 85, 86, 110, Park 8 and *C. hoffmanni* were each tested at various voltages. Tables 3, 4, 5, 6, 7 and 8 show the observed velocities in μ per second and μ per second per volt for these cultures of virulent and avirulent corynebacteria.

TABLE 7

Culture C. hoffmanni

VOLTAGE	MOBILITY	MOBILITY
	μ/second	μ/second/volt
114	69.3	0.607
100	68.7	0.687
80	54.4	0.680
60	39.8	0.663
40	24.3	0.607
30	18.4	0.613
13	8.1	0.623
Average		0.640

TABLE 8

Culture Park no. 8

VOLTAGE	MOBILITY	MOBILITY
	μ/second	μ/second/volt
100	43.5	0.435
60	22.0	0.367
40	12.8	0.322
30	8.2	0.366
20	6.0	0.334
13±	3.1	0.420
Average		0.374

It may be recalled that[3]

$$\text{P.D.} = \frac{4\pi\eta v}{KX} \qquad (1)$$

[3] This is the abbreviated form of the Helmholtz-Lamb equation (cf. Winslow, Falk and Caulfield: Jour. Gen. Physiol., 1923, **6**, 177). According to Debye and Hückel (Physik. Zeitschr., 1924, **25**, 49) instead of 4π, for spherical particles the formula should include 6π. With this correction, P.D. as a ζ (zeta) potential becomes equal to P.D. as the θ potential deduced from the Stoke's law (cf. Harkins: Atoms, Ions, Salts and Surfaces, in The Newer Knowledge of Bacteriology and Immunology, edited by Jordan and Falk, Chicago, 1928).

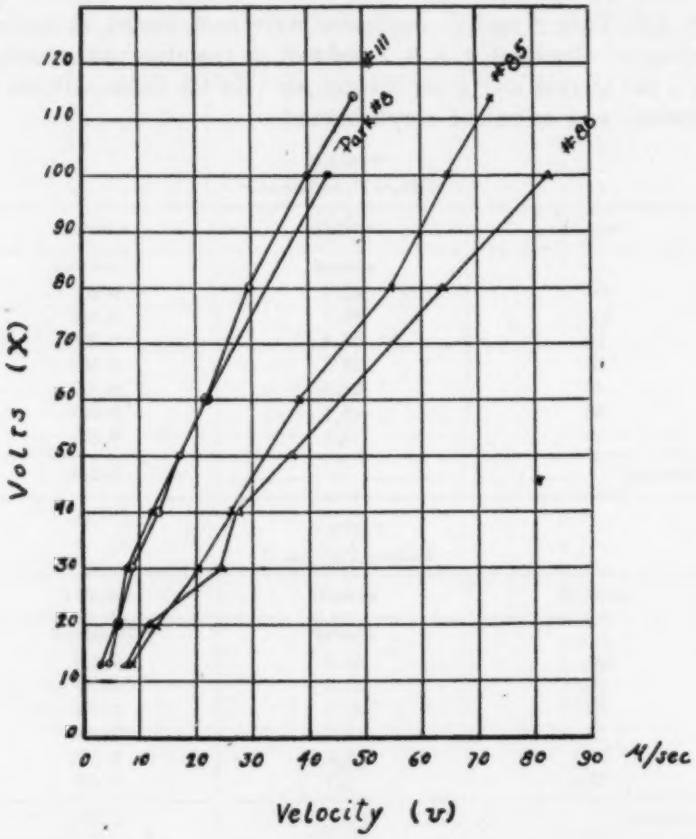

FIG. 4

For suspensions of a given nature in a particular menstruum

$$\text{P.D.} = k \cdot \frac{v}{X} \tag{2}$$

where k is a constant $\left(=\frac{4\pi\eta}{K}\right)$, v is electrophoretic velocity in centimeters per second, X is the impressed voltage in c. g. s. units.

It is obvious from equation (2) for any given suspension (P.D. and k being independent of v and of X and being constant) that:

$$v \propto X \tag{3}$$

Hence, plotting mobility against voltage for a given washed culture a straight line should be obtained if true electrophoresis is observed. Figure 4 shows graphically the results of some of the experiments listed in tables 3 to 8.

TABLE 9

110 volts—4 ocular units (circa 140μ)

CULTURE	MEAN MOBILITIES			VIRULENCE OR TOXIGENICITY (OLD TESTS)
	Seconds/140μ	μ/second/110 volts	μ/second/volt	
Park no. 8	3.4	41.2	0.375	+
No. 85	2.8	50.0	0.455	+
Hoffmanni	2.2	63.6	0.578	−
No. 111	1.9	73.7	0.670	−

TABLE 10

Three-times-washed forty-eight-hour broth cultures

CULTURE	MOBILITY		VIRULENCE
	μ/second/25 volts	μ/second/volt	
Park no. 8	2.6	0.104	+
No. 85	4.1	0.164	+
Hoffmanni	5.0	0.200	−
No. 89	6.6	0.264	−
No. 103	7.2	0.288	−

Measurements were made on once-washed suspensions from four-day Witte's peptone veal broth. The whole broth cultures had been kept in the ice chest for forty-eight-hours. The results of these readings are shown in tables 9 and 10.

It is apparent from tables 9 and 10 that electrophoresis in this simplified cell affords a method for determination of virulence or toxigenicity of diphtheria bacilli.

A larger series of cultures was tested after forty-eight hour

growth in 2 per cent Witte's peptone veal broth to which 0.1 per cent glucose had been added. The bacilli were thrown out of the broth and washed three times in distilled water. It will be observed in this experiment (table 11) that cultures nos. 110 and 109 occupy anomalous positions. Subsequent tests revealed that culture no. 109 shows toxigenic powers only after seven days' growth in broth. We believe, however, that most of the anomalous velocities which we observed in these and other preliminary tests were due in part to the inaccurate position of the V_s (stationary layer) caused by not pressing the coverglass tightly on the cell. Subsequent experiments were performed (*a*) with the cover-

TABLE 11

STRAIN	MOBILITIES				AVERAGE	VIRULENCE (OLD TESTS)
	25 volts	50 volts	25 volts	50 volts		
	μ/second	μ/second	μ/second/volt	μ/second/volt		
No. 102	10.5	22.2	0.420	0.444	0.432	+
Park no. 8	10.7	23.3	0.427	0.466	0.447	+
No. 110	11.5	23.1	0.460	0.462	0.461	−
No. 85	11.7	23.3	0.468	0.466	0.467	+
No. 93	13.8	29.1	0.552	0.582	0.568	−
No. 109	19.1	42.2	0.764	0.844	0.804	+ (?)
Hoffmanni	20.4	43.6	0.816	0.872	0.844	−
No. 111	42.4	71.6	1.300	1.430	1.370	−

glass resting on a heavy cushion of water, (b) with the coverglass too tightly pressed so that the glass was slightly warped, (*c*) pressed gently with four points of pressure and surplus water blotted away; all showed wide differences in velocity at the same impressed voltage with the same culture. The true mobility is observed under condition (*c*).

An experiment with a large series of strains is recorded in table 12 to show anomalous potentials observed before we were aware of errors resulting from not properly adjusting the coverglass on the cell. The three-times-washed suspensions were obtained from forty-eight-hour 2 per cent Witte's peptone veal broth cultures and from eighteen-hour growths on glycerol agar slants.

Following the P.D. determinations, toxigenicity tests with the clear supernatant broth were made in guinea pigs, using 3.0,

TABLE 12

CULTURE	P.D.		TOXIGENICITY TESTS				VIRULENCE	P.D. AGAR CULTURE
			3 cc.	1 cc.	0.5 cc.	0.1 cc.		
			Death in days					
	μ/second/85 volts	μ/second/volt	days	days	days	days		μ/second/85 volts
No. 109	7.9	0.32	½	1	1	3	+	15.9
No. 86	8.0	0.32	1½	1½	1½		+	10.7
Park no. 8	11.6	0.46	3	3			+	7.3
No. 93	13.2	0.53					−	17.3
No. 89	14.2	0.57					−	17.0
No. 110	16.2	0.65					−	10.5
No. 88	17.0	0.68					−	15.4
No. 85	17.6	0.70	3				+	11.1
No. 105	19.0	0.76					−	
No. 111	19.0	0.76					−	11.3
No. 95	20.5	0.82					−	13.7
No. 104	21.3	0.85	1½	3		3	+	
Hoffmanni	22.5	0.90					−	
No. 103	23.0	0.92					−	11.8
No. 102	23.3	0.93	1	½	3		+	9.3
No. 92	23.6	0.94					−	15.7

TABLE 13

Retests of virulence of agar slant cultures (eighteen to twenty-four hours) were made with cultures nos. 85, 102 and 104 using antitoxin controls

CULTURES	SUBCUTANEOUS INJECTIONS			
	3 cc.	1 cc.	0.5 cc.	0.1 cc.
	Death in days			
	days	days		
No. 85	6	8		
No. 102	3	Sick		
No. 104	1½	1½		

1.0, 0.5 and 0.1 cc. quantities inoculated subcutaneously. The results are recorded in table 13.

It is apparent that these three cultures were virulent, but killed only those guinea pigs that received 1.0 or 3.0 cc.

A number of cultures obtained from the Chicago Department of Health were tested after two and seven days' incubation. These strains were grown in 2 per cent Witte's peptone veal broth and washed three times in distilled water before each test. The pH of the two-day suspensions was determined colorimetrically. The two-day suspensions had been held in the ice-chest two days after washing. Table 14 shows the results of these tests.

TABLE 14

CULTURE	2-DAY CULTURE		7-DAY CULTURE		2 CC. OF 48 HOURS BROTH SUBCUTANEOUSLY, VIRULENCE
	μ/second/25 volts	pH	μ/second/25 volts	μ/second/volt	
No. 102	4.6	6.2	6.2	0.249	+
No. 95	5.0	6.2			+*
No. 85	6.5	6.2	6.4	0.256	+
No. 104	6.8	6.4	4.5	0.179	+
Park no. 8	7.1	6.6	5.9	0.236	+
No. 109	7.8	6.0	6.2	0.270	+
No. 86	7.9	6.6	8.4	0.337	+
No. 110	8.2	6.2	9.7	0.387	−
No. 88	8.5	6.4	8.7	0.347	−
No. 103	8.9	5.7	12.7	0.509	− (±)†
No. 93	9.7	6.4	9.1	0.365	−
No. 105	9.9	6.4	8.7	0.349	−
No. 89	10.8	6.0			−
Hoffmanni	14.2	6.6	11.8	0.470	−
No. 92	16.5	6.8	14.3	0.572	−

* Negative on first test. Positive on retest. In some experiments this culture is found to give a high velocity and is avirulent.

† This culture is sometimes slightly toxigenic.

This experiment shows that the acidity of the suspensions does not account for the velocities observed. The inverse relationship between electrophoretic mobility and virulence, i.e., high mobility and avirulence or non-toxigenic bacilli, and low mobility and toxigenicity or virulence is demonstrated in these tests. The dividing line between virulent and avirulent strains in this test is 7.9 to 8.2 for forty-eight-hour cultures and 8.4 to 8.7 for seven-day cultures. The strains giving the intermediate measurements

had been isolated from middle ear infections, scarlet fever and other unusual sources and conditions. They were purposely chosen to show the limitations or values of this method of testing virulence. Guinea pig tests for virulence and toxigenicity have not been more regular than electrophoresis with these intermediate strains: Cultures Nos. 88, 110 and 103 have been found to be slightly toxigenic in some tests when 3 cc. of seven-day broth cultures were injected into guinea pigs and at other times have been non-toxigenic under conditions supposedly similar.

TABLE 15

48-HOUR BROTH CULTURE	TIME FOR COMPLETE PRECIPITATION	VIRULENCE	P.D. (μ/SECOND/25 VOLTS)
	minutes		
No. 102	2	+	4.6
No. 95	2	+	5.0
No. 85	2	+	6.5
No. 104	20	+	6.8
No. 109	2	+	7.8
Park no. 8	2	+	7.1
No. 86	20	+	7.9
No. 110	20	−	8.2
No. 88	20	−	8.5
No. 103	20	±	8.9
No. 95	20	±	5.0
No. 92	20	−	16.5
Hoffmanni	More than 20	−	14.2

However, the electrophoretic mobility has varied in a correlated manner with these variations.

An experiment was designed to show the stability of suspension in the second change of distilled water and correlation with virulence and electrophoretic mobility. Table 15 indicates that there is a relationship between the time to precipitate the cells at 1500 r.p.m. and virulence with the concomitant mobility, although other factors undoubtedly enter.

Table 16 shows the results of testing seven-day, three-times-washed bacilli from broth cultures, making the P.D. measurements with the small apparatus.

Seven-day-old cultures of our collection are readily identified

by the electrophoresis method of determining the toxigenicity of corynebacteria. In a preceding paper it was shown that certain toxigenic strains did not produce demonstrable toxin (by guinea pig method) until three, four, and five or more days. Conversely, certain strains (culture No. 19 described as a type) produce their maximum toxigenicity after a week's growth. It was shown that

TABLE 16

CULTURE	μ/SECOND/25 VOLTS	TOXIGENICITY TESTS, 2 CC. SUBCUTANEOUSLY	VIRULENCE
No. 102	Motionless	Died 40 hours	+
Park no. 8	0.5	Died 18 hours	+
No. 109	1.8	Died 4 days	+
No. 85	Motionless	Died 72 hours	+
No. 105	2.2	Sick 18 hours, died 7 days	±
No. 95	4.6	Died 40 hours	−
No. 103	5.5	Survived*	±
No. 88	19.5	Survived	−
Hoffmanni	20.0	Survived	−

* Seven-day culture killed test animal in twenty-four hours in other tests.

TABLE 17

CULTURE	SMALL APPARATUS SUSPENSIONS	TOXIGENICITY TESTS, 2 CC. INJECTED SUBCUTANEOUSLY
	μ/second/25 volts	
No. 85	1.6	Died 1 day
No. 102	2.2	Died 1 day
Park no. 8	3.2	Died 18 hours
No. 95	3.3	Died 2 days
No. 109	(4.7) (6.8)	Died 4 days
No. 88	7.9	Survived
No. 105	11.0	Sick 18 hours—survived
Hoffmanni	19.0	
No. 103	21.2	

the mobility curve varied inversely with that of toxigenicity. Hence, it is possible that a few strains may escape identification by this method of measuring the mobility of three-times-washed bacilli suspended in distilled water. However, these equivocal results are less apt to be obtained when mobilities of bacilli in whole broth cultures are measured in the large apparatus. *It*

must be repeated that standard guinea pig tests for toxigenicity and virulence of borderline strains are no more accurate than these physical measurements.

Table 17 represents another experiment on three-times-washed bacilli as well as measurements on whole broth cultures. The bacilli were grown seven days in 2 per cent Witte's peptone veal broth to which 0.1 per cent glucose had been added. Toxigenicity tests were conducted with the clear supernatant broth obtained by centrifugating the whole broth cultures.

From numerous similar experiments it was plain that true electrophoresis measurements are obtainable by use of this simplified cell if the coverglass is fitted properly and three-times-washed cultures are used as test suspensions.

The data show that toxigenic diphtheria bacilli may be identified by the method.

IDENTIFICATION OF UNKNOWN DIPHTHERIA CULTURES FOR THE CHICAGO DEPARTMENT OF HEALTH BY USE OF THE SIMPLE ELECTROPHORESIS CELL (P.D. OF WASHED SUSPENSIONS)

It has been shown that there were significant differences in electrophoretic mobilities between toxigenic and non-toxigenic corynebacteria. These potential differences were found when measurements were made on diphtheria bacilli in the menstrua in which they were grown or on three-times-washed bacilli suspended in distilled water. It was found that cultures tested in this way must be incubated at least forty-eight hours before unequivocal results may be obtained. This is also the case when measurements are made on distilled water suspensions of bacilli. In some instances a culture did not show low mobility until after a seven-day incubation period.

The work reported in this section is an actual demonstration of the reliability of this method for identifying diphtheria bacilli in the Department of Health laboratory. One hundred and fifty-three cultures of corynebacteria were obtained from forty-nine sources during an outbreak of diphtheria in a Chicago Public School. The virulence of each of these strains was determined

by the Bureau of Laboratories and Research of the Chicago Department of Health, using the guinea pig test for virulence. At the same time transplants of these cultures were given to us. The city laboratory findings were unknown to us until after the completion of the electrophoresis experiments.

Our cultures were grown in veal infusion, 2 per cent Witte peptone broth, pH 7.2, to which 0.1 per cent glucose had been added. Approximately 50 cc. of broth were placed in 250 cc. flat bottom flasks and sterilized. After two- and seven-day incubation periods at 37°C. about 10 cc. of the whole broth culture were centrifugated at 2000 r.p.m. for several minutes and the sediment of bacilli resuspended in distilled water and washed three times with distilled water. Mobilities were then determined in the simplified cell, using 48 volts D.C.

Table 18 shows P.D. observed after two- and seven-day incubation periods and the reports from the Chicago Department of Health. It will be observed that our physical measurements detected 100 per cent of the "*C. hoffmanni*" strains, and 100 per cent of the "pleomorphic granular short bacilli." Two cultures of "multigranular long bacilli" were called virulent by the electrophoresis method but were found to be avirulent by the Chicago Department of Health guinea pig tests. We were 94.4 per cent accurate in detecting "typical *C. diphtheriae.*" We called culture 7118B avirulent. Upon retesting by the animal virulence test this strain proved to be virulent. With this one exception, our errors were on the side of safety because we called those strains virulent which were found to be avirulent on retests by the Department of Health.

At this point the characteristics of the slide cell method may be recapitulated:

1. The apparatus is simple.
2. The electrophoretic measurement is made quickly and easily.
3. It demands the use of a small quantity of three-times-washed suspensions from broth cultures at least forty-eight hours old.
4. Unless older cultures are used, strains which in culture pro-

TABLE 18

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O, P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
7537–2	–	–	20.0	21.2
–4	–	–	13.6	22.6
–5	–	–	19.3	20.0
7494–1	+	+	14.8	4.8
–2	+	+	19.3	7.6
–4	+	+	6.1	10.6
–5	+	+	11.3	11.3
7593	–	–	19.3	13.6
7579–2	–	–	18.7	19.3
–5	–	–	28.3	12.3
7470–4	–	–	22.6	17.0
7578	+	+	(4.2) 6.8	8.5
7118–1	+	+	4.0	13.8
–2 A	+	+	7.4	9.7
–3	+	+	6.8	17.0
–4 B	+	–	19.3	18.8
–5	+	–	19.3	17.0
7156–1	+	+	10.0	9.7
–2	+	+	16.0	9.1
–3	+	+	11.3	5.6
–4	+	+	13.6	6.6
–5	+	+	13.6	7.6
7180–5	–	–	20.0	17.0
7110–1	+	+	6.8	9.1
–3	+	+	10.9	10.9
–4	+	+	6.8	11.3
–5	+	+	19.3	8.5

* If a mobility measurement as low as (or lower than) 11.3 appeared, the culture was called "virulent."

TABLE 18—*Continued*

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O, P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
7264–1	+	+	5.7	8.5
–2	+	+	4.3	12.6
–3	+	+	6.8	7.9
–4	+	+	4.6	6.2
–5	+	+	4.3	10.9
7278–4	+	+	4.6	11.3
7245–1	+	+	3.6	9.1
–2	+	+	9.7	13.6
–4†	+	+	11.3	11.3
–5	+	+	13.6	9.7
7508–5	–	–	20.0	20.0
–6	–	–	26.0	13.6
–7	–	–	26.0	26.0
7476–1	+	+	8.5	11.3
–2	+	+	8.5	7.6
–3	+	+	12.6	7.6
–4	+	+	5.8	11.3
–5	+	+	9.7	7.6
7295–5	–	–	19.3	13.6
7108–1	+	+	5.2	6.1
–2†	+	+		
–3	+	+	8.5	7.6
–4	+	+	26.0	6.2
–5	+	+	11.3	7.6
7216–1	–	–	13.5	17.0
–2	–	–	26.0	13.6
7194–1	–	–	34.0	34.0
–2	–	–	34.0	17.0
–3	–	–	34.6	20.0
–4	–	–	18.7	28.3
–5	–	+	11.3	8.3

† Contamination.

TABLE 18—*Continued*

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O. P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
7533–3	+	+	8.5	11.3
–4	+	±	19.3	11.3
7169–2	+	+	6.2	9.1
7468–4	–	–	19.3	17.0
7137–2	+	+	4.2	9.7
7329–1	–	±	20.2	8.5
–3	–	+	11.3	6.8
–4	–	±	15.4	13.6
7514–2	–	–	11.3	17.0
–5	–	+	9.7	6.5
8381–1	+	+	6.2	6.8
–2	+	+	17.0	11.3
–3	+	+	6.2	6.8
7740–2	+	+	7.5	8.5
–3	+	+	16.1	4.2
7772–1	+	+	10.9	7.7
–4	+	+	6.8	7.7
7666–3	+	+	9.7	11.3
–4	+	+	4.9	3.7
7597–3	+	+	7.5	6.8
–4	+	+	9.7	6.8
7744–1	+	+	8.1	11.3
–2	+	+	8.1	11.3
–3	+	+	6.8	11.3
–4	+	+	10.9	4.2
–5	+	+	11.3	4.2
7806–2	+	+	11.3	9.4
–3‡	+	–	22.6	34.0
–4	+	+	8.5	4.2

‡ Yellow colony.

TABLE 18—*Continued*

HEALTH DEPARTMENT NUMBER	VIRULENCE[a] ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O, P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
7825–2	−	−	15.6	34.0
–4	−	−	17.0	64.0
–5	−	−	18.5	11.3
7666–4	−	−	17.0	34.0
–5	−	−	17.0	34.0
7812–1	+	+	7.5	9.6
–2	+	+	20.0	10.0
–4	+	+	8.5	9.6
7658–1	−	−	18.5	34.0
–2	−	−	15.6	34.0
8394–A1	−	+	6.5	11.3
–A4	−	+	3.7	4.1
–A5	−	+	10.6	9.7
–B2	−	+	7.7	6.5
7677–1	+	+	8.5	7.6
–4	+	+	11.3	4.8
–5	+	+	7.2	7.6
7717–1	+	+	2.2	3.7
–2	+	+	4.2	3.7
–3	+	+	9.7	4.2
–4	+	+	17.0	12.5
–5	+	+	10.3	3.5
8350–1	+	+	8.5	8.1
–2	+	+	17.0	5.0
7780–1	−	+	14.0	9.7
–2	−	+	6.8	7.5
–3	−	+	2.2	11.3
–5	−	+	4.2	4.2
8939–1	−	+	10.0	10.0
–2	−	+	3.7	6.8
–3	−	+	3.7	6.8
–4	−	+	8.5	6.8
–5	−	+	48.0	15.6

TABLE 18—*Continued*

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O, P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
7244–1	−	+	6.8	6.4
–2	−	−	10.3	8.5
–3	−	−	22.6	8.5
–4	−	+	6.8	6.2
–5	−	−	17.0	6.2
7279–1	+	+	8.1	7.6
–2	+	+	7.5	5.6
–3	+	+	11.3	12.6
–4	+	+	12.5	10.9
–5	+	+	6.8	5.6
7196–1	+	+	11.3	6.8
–2	+	+	17.0	6.8
–3	+	+	8.7	4.2
7332–1	+	+	6.8	7.6
–2	+	+	6.8	7.6
–3	+	+	8.5	9.7
–4	+	+	6.8	7.6
–5	+	+	6.8	7.6
7208–1	+	+	4.8	6.8
–2§	+	+	13.6	34.0
–3	+	+	9.7	34.0
–4	+	+	4.9	6.8
–5	+	+	6.2	6.8
7374–1	+	+	2.2	2.0
–2	+	+	6.2	6.8
–3	+	+	6.2	6.8
–4	+	+	7.5	6.8
–5	+	+	6.8	11.3
7371–1	+	+	12.5	4.4
–2	+	+	6.8	6.8
–3	+	+	7.5	10.9
–4	+	+	6.8	6.5
–5	+	+	6.8	6.5

§ Mixed yellow colony.

TABLE 18—*Continued*

HEALTH DEPARTMENT NUMBER	VIRULENCE* ACCORDING TO		THREE TIMES WASHED IN DISTILLED H_2O. P.D.	
	Health department tests	Electrophoresis (11.3 as dividing value)	Forty-eight-hour	Seven-day
			μ/second/48 volts	*μ/second/48 volts*
Park no. 8	+	+	2.9	7.6
	+	+	6.5	8.0
	+	+	2.9	9.1
	+	+	4.2	3.5
	+	+	2.9	7.6
Hoffmanni	−	−	19.3	34.0
	−	−	19.3	64.0
	−	−	14.0	34.0
	−	−	34.0	34.0

duce toxin slowly (i.e. seven days) will frequently be reported as "nonvirulent."

5. In general, it gives results which when checked against guinea pig tests are reliable.

The details of the technique are given below. It will be noted that a further manipulative disadvantage of the method is the necessity for making the mobility measurements with the microscope focussed on the "stationary level."

To meet certain obvious objections to these points in technique, we undertook a series of experiments to develop an apparatus which would (*a*) permit the use of growth taken directly from the original Loeffler medium; (*b*) eliminate the necessity for even forty-eight-hour subculturing, and the washing of the cultures, and (*c*) eliminate the necessity for focussing in the "stationary level." To these ends, we have developed the "capillary tube" method.

It may be recalled that when electrophoresis is conducted in a closed system, the electrical double layers between the fluid and the walls (glass) are located in the positions indicated in the schematic diagram of figure 5. Under the influence of the applied electrical field, the (positive) electrical layers in the water are given a positive velocity to the cathode and drag the successively

contiguous layers of water along at lesser and lesser velocities. The return streaming of the water occurs in the middle of the cell (if it is symmetrical). Hence the electrophoretic velocities of suspended particles vary at different levels in the cell and the "true" velocity is observed only at the position of the "stationary level" of the fluid. For this reason the mobility measurements are made with the microscope focussed at the "stationary level." Mooney[4] has pointed out that these effects of the endosmotic streaming of the fluid upon the electrophoretic mobility may be eliminated by using a cell of capillary dimensions. The principle

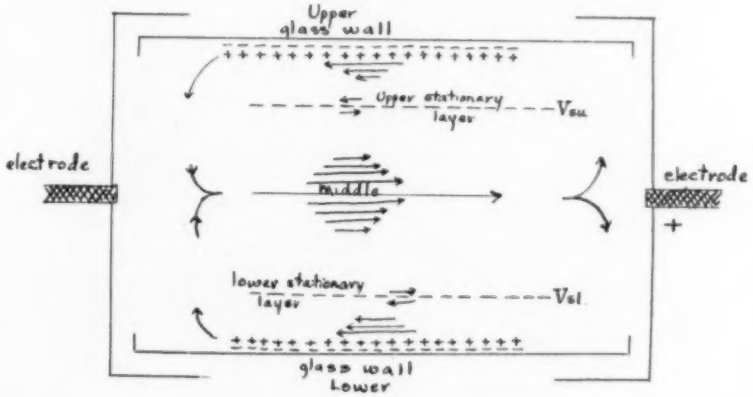

FIG. 5

of the method is that the electrical double layers at the fluid-wall interphase are brought so close together that, by the viscous resistance of the fluid, they drag all of the fluid along at approximately the same velocity. Applied to the electrophoresis of bacteria, the situation is this: Under the influence of the external electrical field, all of the fluid (water) in a glass capillary tube moves in one direction (to the cathode) at a velocity which is practically without a gradient across the diameter of the tube. Bacteria suspended in the water, being electronegatively charged, tend to move in the opposite direction to the anode. Hence, under any

[4] Phys. Rev., 1927, **29**, 218.

given set of conditions *the observed velocity of the bacteria is the difference between the velocity of the endosmotic streaming of the water* (*End.*$_w$) *and the velocity of electrophoresis of the bacterium* (*Elect.*$_b$). Thus:

$$\text{Observed mobility} = \text{End.}_w - \text{Elect.}_b$$

In a series of measurements, if the necessary precautions are taken, End$_w$ is sensibly constant. Hence, *the observed mobility of the particle* (*bacterium*) *varies inversely as its true mobility*, and is

FIG. 6. THE CELL USED IN THE CAPILLARY TUBE METHOD

approximately independent of its position in the capillary tube. Furthermore, by the simple arrangement of apparatus shown in figure 6, polarization at the electrodes is practically eliminated by the interposition of distilled water between the electrodes and the open ends of the capillary tube.

With the capillary tube apparatus, the technique is as follows:

1. An isolated colony with the characteristics of the diphtheria bacillus is picked from the original Loeffler's medium growth with a *clean* loop.

2. It is emulsified in a small quantity of water in a clean crystal.

3. A loopful of this suspension is used for the routine microscopical examination to detect bacilli with the morphology (shape, size, granular, barred, solid, etc. characteristics) of diphtheria bacilli.

4. If the microscopical examination is "positive," a clean capillary tube is filled with the suspension (by capillarity) and is used for the electrophoretic measurement.

The details of the apparatus and technique are given below.

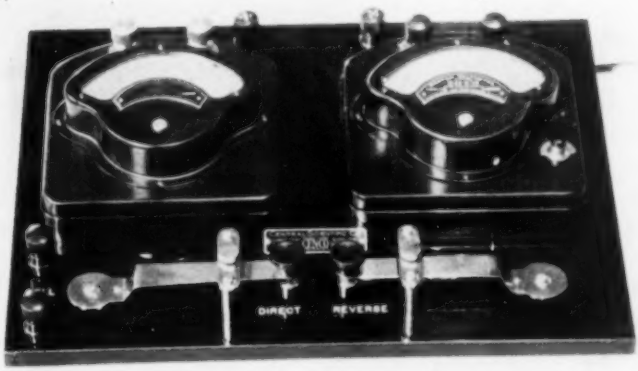

FIG. 7. A CONVENIENT ARRANGEMENT OF THE ELECTRICAL APPARATUS FOR ELECTROPHORESIS

DETAILS OF TECHNIQUE

A. The slide cell method

1. Cultures. If pure cultures are already available they should be grown in veal infusion, 2 per cent Witte peptone broth, initial pH = 7.2 to 7.4, at 37°C., preferably for at least forty-eight hours. If original cultures from throat, nose, etc., on Loeffler's medium are to be used, three to six separate colonies should be transferred to broth and used as for pure cultures. Sediment the bacteria by centrifugation; resuspend in distilled water and repeat twice more. Use these three-times-washed organisms in

distilled water in a suspension that is definitely cloudy to the naked eye. The distilled water suspension should have a pH near 7.0.

2. Cell.[5] Clean by brushing with soap and water and rinsing under running tap, and then, distilled water. Fill with suspension nearly to overflowing with a fine tipped pipette. Place coverglass over cell without trapping air bubbles. Press down coverglass so that it rests on the glass and not merely on the fluid in the cell. Wipe up excess fluid with filter paper. Connect electrodes to source of direct current voltage and place the cell on the stage of the microscope so that the objective is over the central portion of the cell.

3. Source of voltage. Any direct current source may be used. If the lighting circuit is D.C. this is satisfactory. A rheostat may be used to reduce the voltage applied to the cell electrodes. It is preferable to use ordinary radio "B" batteries, from which 25 or 45 to 50 volts can be taken. An impressed voltage of 25 to 50 volts (preferably the higher voltage) is satisfactory.

4. Microscope and equipment. Any microscope with a calibrated head on the fine adjustment screw will serve. We have found the following optical system satisfactory: objective—Bausch and Lomb, 8 mm., 0.50 n.a., 21× (long focal working distance); ocular—Bausch and Lomb, 25×, compensating; ocular micrometer—Sedgwick-Rafter ruling, placed over the diaphragm of the ocular. (Any similar equipment will serve.) Calibrate the ocular micrometer with a stage micrometer so that the diameter of each large square in the ocular micrometer is known in micra. Have available a good stop-watch reading to a fifth of a second.

5. Calibration of the cell. Fill the cell with water, cover with the coverglass, bring the calibrated head of the fine adjustment screw to zero and focus the microscope on the bottom of the chamber in the cell with the coarse adjustment. By turning the fine adjustment head until the microscope is focussed on the bottom of the coverglass, determined the depth of the cell chamber

[5] The cells and accessory equipment are made according to our specifications by the Central Scientific Company of Chicago.

in units on the head of the fine adjustment screw. Using the formula given below, focus the microscope on the upper or lower "stationary level." Do not change this focus.

In a cell of the kind described here, the stationary layers are located at a distance of $1/2\ D \div \sqrt{3}$ above and below the middle of the cell. Thus, if a cell is 100 units deep on the micrometer screw head, the middle is 50 units; $1/2\ D = 1/2$ depth $= 50$; and $50 \div \sqrt{3}$ is 28.9; the lower stationary level (*Vsl*) is located at 21.1 units from the bottom of the cell and the upper one (*Vsu*) at 78.9 units from the bottom. These levels, determined for each cell, are the ones at which the electrophoresis measurements are made.

6. Illumination. Natural or artificial illumination should be adjusted as for hanging drop examinations.

7. Electrical connections. The cell should be wired to the source of voltage through a reversing switch so that the polarity of the electrodes can be reversed quickly, easily and without jarring or moving the cell. It is desirable to have a small ammeter and a small voltmeter in the circuit to provide direct checks on the circuit. The voltmeter should show that the impressed potential is constant and the ammeter that practically no current is flowing (i.e., less than 10 milliamperes) and hence that the suspension in the cell is a very poor conductor. An extra main switch in the circuit, as indicated in our diagram, (fig. 2) is desirable. (Note: All that is *essential* is the cell, a good "B" battery and a reversing switch).[6]

8. Measurements. After the eye has become adjusted to the illumination of the field of vision in the microscope, the bacteria in clumps or singly should be plainly visible. With one hand on the reversing switch or keys and the other controlling a stopwatch throw on the voltage. As bacteria come into focus, moving across the field of vision, determine the time it takes for them to pass the diameter of one, two or more squares on the ocular micrometer. Make at least five measurements on different bacteria or clumps. Reverse the orientation of the electrical

[6] The Central Scientific Company of Chicago has prepared the electrical circuit in a compact and convenient arrangement. (See fig. 7.)

field by reversing the switch or keys and repeat the measurements with the bacteria moving in the opposite direction. Average the measurements for equal numbers of observations in the two directions. This average measurement is a velocity in "seconds per[7] micra." Calculate velocity in "micra per second." Dismount the cell and clean as before.

B. The capillary tube method

1. Cultures. Pick from the Loeffler's medium which has been incubated twelve to twenty-four hours at 37°C. three to six separate colonies that resemble colonies of diphtheria bacilli. Transfer each colony to a few drops of sterile distilled water on a clean slide or watch glass. Use a loopful of this suspension for the routine microscopical examination. If this is "positive," proceed with the electrophoretic measurement. A satisfactory suspension has a turbidity of about 1 billion organisms per cubic centimeter.

2. Capillary tube. This should be a little longer than the distance between the electrodes and should have an internal diameter of approximately 0.2 to 0.3 mm. (With a little practice capillaries of these internal dimensions can be drawn from heavy wall tubing. If a number of measurements are to be made, it is desirable to have on hand a set of capillaries of the same diameter. With the optical system recommended, the diameter of the capillary is somewhat less than the diameter of the field of vision).[8] The capillary tube is cleaned first with dichromate-sulfuric acid cleaning solution and then with running tap and distilled water. Water may be run through by inserting the end of the capillary in a piece of soft rubber tubing attached to the hub of a 5 cc. hypodermic syringe.

Holding the capillary between clean forceps, let it fill with the test suspension in the watch glass by capillarity. The cell should be filled with distilled water to a definite level (marked), constant in all measurements, and the capillary laid across the

[7] The value here is determined by the stage micrometer equivalents of the ocular micrometer units and by the number of ocular micrometer unit squares across which the bacteria moved in each measurement.

[8] The cell and capillary tubes are supplied by the Central Scientific Company of Chicago.

electrodes with the ends projecting the same distance beyond the two electrodes.

3. The source of voltage, the electrical circuits, connections, etc., and the optical system are the same as for the slide cell method described above.[9]

4. Measurements. Focus the microscope on the middle of the interior of the capillary. Apply the external voltage and record the velocity of movement of a bacterium. *Immediately*, reverse the polarity and repeat a measurement. Repeat this process until 6 to 10 measurements have been obtained. Convert the measurements to velocities in micra per second (μ/sec.).

Caution. It is recommended that these methods should be used by Schick-negative individuals only.

STANDARDIZATION OF ELECTROPHORESIS MEASUREMENTS

Instead of presenting specific recommendations on the standardization of electrophoretic measurements against guinea pig tests, we prefer to list the following which—though they recommend departures in principle from certain accepted procedures—we believe are theoretically as well as technically correct.

1. When standardizing the electrophoretic methods, first make measurements with pure cultures (prepared as indicated above) known by quantitative guinea pig tests to be (*a*) highly toxigenic; (*b*) moderately toxigenic, and (*c*) non-toxigenic.

2. It should be found that for such a series of cultures the electrophoretic velocities fall in a sequence which (*a*) with the slide cell method is an increasing velocity sequence, and (*b*) with the capillary tube method is a decreasing velocity sequence.

3. In routine practice, with each series of "unknown" cultures examined, there should be used as controls at least three cultures known to be highly-, moderately- and non-toxigenic, respectively. (We have found that with a little practice, some 12 to 20 slide cell and some 10 to 15 capillary tube measurements can be made in an hour, particularly if a helper is available to clean apparatus between measurements, or if several clean cells or capillary tubes of the same dimensions are prepared in advance.)

[9] A water immersion lens is perhaps preferable to the one already specified (i.e., Bausch and Lomb 7 mm. "water," 0.50 n.a.).

4. In reporting virulence (toxigenicity) results from electrophoresis measurements, cultures or suspensions with extremely high or extremely low velocities should be reported as a "negative" or "positive" (according to which method of measurement is used); and cultures with intermediate velocities should be reported as + (−) or − (+) according to which velocity extreme they approach, and subject to reexamination by an electrophoretic method or by a suitable quantitative guinea pig test.

When multiple measurements of several colonies (or cultures from several colonies) taken from a throat, nose or other streak culture show significantly different results, the diagnosis should be determined by that set of measurements which gives velocities most nearly approaching the velocity of cultures known to be highly virulent (toxigenic).

5. The last preceding statement applies as well to measurements made on suspensions or cultures which are apparently or certainly mixed or contaminated.

In conclusion, we would emphasize that we have presented here a series of presumptive methods. It is hoped that workers in other laboratories will experiment with them. They may find, as we have found, that they are useful and reliable in providing rapid, tentative "virulence diagnoses" without using animals, without waiting days for the results of animal tests and where animal tests are inconvenient or impossible. Further experimentation may bring to light shortcomings of the techniques that may be avoidable. The values of the methods can be determined only as the results of wider experience become available.

Finally, we would add that the studies summarized in this series of papers may be useful in research as well as in routine observations, particularly because they provide significant evidences on apparent quantitative gradations in toxigenicity. It may not be gratuitous to remark that, in our opinion, the older qualitative notion has dictated practices which have resulted in missing many "virulent" strains of diphtheria bacilli and in complicating diphtheria epidemiology to an extent that may have been significant in the failure of epidemiological methods in the past to effect control of diphtheria incidence and morbidity.

The simplified electrophoresis methods described here may also be useful in other fields of study.

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THE Council of the Society has authorized the establishment of a directory where *those who desire to fill vacancies* may secure a selected list of applicants. This list is composed of teachers of bacteriology, technicians in laboratories, applicants for fellowships and scholarships in bacteriology, serology, immunology, and research workers in medical or agricultural bacteriology. Employers may receive information regarding applicants without charge. Information regarding vacancies should be filed upon a blank which has been prepared to secure uniform information regarding a particular vacancy.

Apply for application blanks at the following address: F. M. MEADER, M.D., *Detroit Department of Health, Detroit, Mich.*

Employment Agency Society of American Bacteriologists

THE Council of the Society has authorized the establishment of an employment agency. Bacteriologists and technicians may learn of positions by making application to the address below. Applicants for positions if not members of the Society will agree to send to the agency 5% of their salary for the first three months. If the applicant is a member of the Society he will agree to send only 3% of his salary for the first three months.

All funds accruing to the Society in excess of necessary and actual expenses, after being audited by the Society will be transferred to the treasurer of the Society for the improvement and enlargement of its Journal, support of its research fellowship, collection of cultures of bacteria, etc.

Send for application blanks to F. M. MEADER, M.D., *Detroit Department of Health, Detroit, Mich.*

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